Patterning of dendritic territories by dendrite-dendrite and dendrite-substrate interactions

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ABSTRACT

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Dendrites develop in highly complex environments and their interactions with neighboring neurons and the substrate are thought to be important for the establishment of their dendritic territories. Mechanisms required for the establishment of dendritic territories remain largely elusive. This thesis investigates the role of both dendrite-dendrite interactions and dendrite-substrate interactions in determining dendritic boundaries. As a part of this study I have uncovered novel mechanisms that neurons utilize to define dendritic borders and how those borders may be important determinants of neuronal function. The experiments described in this thesis have taken advantage of genetic tools in *Drosophila* that allow manipulation and visualization of individual neurons.

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Introduction

Nervous systems are comprised of a vast number of neuronal types with characteristic dendritic morphologies and axonal projections that determine their synaptic partners and function in neuronal circuits. Dendrite arborization patterns of neurons are a hallmark of neuronal type. The shape, size and the territories of dendrites are primary determinants of function as they influence the range of stimuli a neuron receives and determines its interaction with neighboring neurons (Corty et al, 2009; Jan & Jan, 2010; Wassle et al, 1981a). By understanding the underlying principles that control the development of dendritic arbors we can shed light on how neuronal morphological and function diversity is generated during development. These insights will ultimately be critical for deciphering the relationship between neuronal form and function(Jan & Jan, 2010; MacNeil & Masland, 1998) and may further provide insights into the basis for aberrant developmental trajectories that contribute to neurological disease.

How dendritic arbors take their final shape has been the subject of rigorous study in the past decade. Although we have uncovered numerous signaling pathways and transcription factors that regulate dendrite morphogenesis, mechanisms regulating the final shape of dendrites remain largely undetermined. The formation of dendritic fields is a complex process that occurs through the action of genes that promote periods of dendritic extension, branching, guidance and, finally, termination of growth. Different classes of molecules appear to play distinct roles in the regulation of dendritic development. For example, transcription factors play an important role in specifying neuronal identity and regulate genes that control cell-type specific dendritic branching and complexity. The degree of dendrite branching is additionally under the regulation of

growth factors such as neurotrophin 3, brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF). Extrinsic guidance factors play an important role in determining the shape and size of dendritic arbors as they guide dendrites to appropriate areas. In some cases, dendrite-dendrite interactions with neighboring cells may determine the final area that is occupied by dendrites of neurons. In addition the final pattern of the dendritic field may be further refined by remodeling and pruning events. I will discuss the molecular pathways that have been shown to control dendrite development in more detail below.

The control of dendritic growth also critically requires interactions with the extracellular environment. Growth promoting cues and cues that promote maintenance of dendrites can be provided by surrounding tissues to help ensure complete innervation of territories. In contrast, growth-inhibiting mechanisms prevent innervation of regions that are incompatible with proper function, such as the territories occupied by other cells of the same type.

The pattern of dendritic fields is therefore influenced by a myriad of intersecting pathways that include cell surface receptors, cell recognition molecules, guidance cues and substrate interactions, only a small fraction of which have been characterized to date.

The *Drosophila* peripheral nervous system (PNS): a model system for studying dendritic patterning

Studies in the *Drosophila melanogaster* PNS have provided important insights into neuronal cell fate specification and differentiation. A major strength of this system is the

opportunity it provides to visualize and manipulate individual neurons with defined dendritic patterns. These technical advances in the past decade have provided tools to investigate the molecular basis of PNS dendrite patterning with extraordinary resolution and specificity, leading to the identification of pathways that play conserved roles across evolution.

Body wall sensory neurons can be divided into three morphological types: chordotonal (ch) organs, external sensory (es) organs and multidendritic (md) neurons (Bodmer et al, 1987; Grueber et al, 2002). The md neurons comprise three distinct neuronal populations; the bipolar dendrite (bd) neurons, tracheal dendrite (td) neurons and dendritic arborization (da) neurons. The dendrite patterns of td and bd neurons are relatively simple, however da neurons show distinct branching patterns that range from simple (approximately 20 branches per cell) to highly complex (over 700 branches per cells). Each abdominal segment in the larvae is innervated by fifteen da neurons, which have been categorized into four classes on the basis of these distinct branching patterns. (Grueber et al, 2002) The conspicuous differences in branching pattern between the classes, as well as the ease with which they can be genetically manipulated and imaged makes da neurons an ideal system for investigating molecular mechanisms that control the diversity of dendrite branching, tiling and targeting (Grueber et al, 2002; Grueber & Jan, 2004; Jan & Jan, 2010; Matthews & Grueber, 2011; Shimono et al, 2009; Yasunaga et al, 2010).

Regulation of shape and organization of dendritic fields by dendro-dendritic interactions

The arrangement and spacing of dendritic branches of a given neuron ultimately determines the space that they sample and neurons they connect with. Innervation of appropriate input regions is essential for the function of sensory neurons in particular as this ensures proper sampling of the sensory world. Multiple mechanisms act in concert to regulate the spatial arrangement of branches within a dendritic arbor, including intrinsic and extrinsic signals that influence the formation, spread, maintenance and remodeling of dendritic territories. Two key cellular mechanisms that control the spatial patterning of dendritic branches are self-avoidance and tiling.

Self-avoidance

For a neuron to effectively sample its environment it must occupy its territory efficiently and without gaps or redundancy (Lawrence Zipursky & Grueber, 2013). In order to ensure that territories are fully innervated, dendrites must grow to an appropriate length and branches must spread to create a pattern that lacks major gaps in coverage. One way in which dendrites accomplish this is by self-avoidance. Self-avoidance refers to the general tendency of branches within a dendritic arbor to avoid overlapping with each other. As a result, dendrites are spread uniformly across their field to effectively sample incoming information (Kramer & Kuwada, 1983). Self avoidance was first described in leech somatosensory neurons (Kramer et al, 1985). Early experimental studies of self-avoidance showed that ablation of one axonal branch leads to the growth of neighboring

axonal branches into the vacated space (Kramer et al, 1985). This result suggested that branches belonging to the same neuron recognize each other, and that this recognition normally leads to repulsion between sister branches (Kramer et al, 1985).

How is recognition and repulsion controlled at the molecular level? Several decades after these initial experiments, studies have uncovered the molecular basis of self-avoidance both in invertebrate and vertebrate systems. In *Drosophila*, *Down syndrome cell adhesion molecule 1* (*Dscam1*) is a key component regulating self-avoidance (Hughes et al, 2007; Matthews et al, 2007; Soba et al, 2007). Dscam1 encodes an Ig domain cell surface receptor, which as a result of alternative splicing can give rise to approximately 19,008 different proteins with unique ectodomains (Schmucker et al, 2000). Binding assays have shown that identical ectodomains bind to one another and not to ectodomains of other isoforms (Wojtowicz et al, 2004; Wojtowicz et al, 2007), in vivo experiments performed by expressing a single Dscam1 isoform demonstrated that homophilic interactions result in repulsion (Hughes et al, 2007; Matthews et al, 2007; Soba et al, 2007). Repulsion upon homophilic binding promotes avoidance between sister dendrites (Hughes et al, 2007; Matthews et al, 2007; Soba et al, 2007). Furthermore, stochastic expression of numerous Dscam1 isoforms presented on the surface of each neuron, a combination that non-self neurons cannot recognize, results in fields of different neurons co-existing and arborizing across the same space (Corty et al).

Starburst amacrine cells in mice use very similar mechanisms to mediate dendritic self-avoidance. In mammals, the clustered protocadherins (Pcdh) encode a class of highly diverse transmembrane proteins that consists of three exon regions Pcdh α, Pcdh β and

Pcdh γ that can each generate multiple splice variants (Hirayama et al, 2012). Pcdh diversity is generated through alternate promoter choice, which permits discrimination of self and non-self. Knockout of 22 Pcdh genes within the γ-cluster leads to extensive dendrite overlap, and a single isoform is sufficient for self-recognition and repulsion (Lefebvre et al, 2012). Further, similar to *Drosophila*, misexpression of a single isoform in neighboring neurons disrupts the normal co-existence (Lefebvre et al, 2012). Thus, although vertebrates and invertebrates use different molecules to mediate discrimination of self from non-self in neurons, their underlying recognition strategies are strikingly similar (Lawrence Zipursky & Grueber, 2013; Lefebvre et al, 2012).

Tiling

Another important question in dendritic patterning is how dendrites appropriately terminate their growth. One component of growth-restricting behavior studied widely in many systems is tiling. Tiling ensures complete and non-redundant coverage of dendrites among functionally identical neurons. Tiling was first described in mammalian retinal ganglion cells (RGCs) (Devries & Baylor, 1997; Wassle et al, 1981a; Wassle & Riemann, 1978). Early studies of the α ganglion cells described two functionally distinct sub-populations, ON and OFF cells (Wassle et al, 1981b). Further work on the α-ganglion cells showed that only functionally similar cells tended not to overlap so as to cover the entire sensory field completely and with minimal overlap (Vaney, 1994; Wassle et al, 1981a). The principle of tiling has been extended to most cell types in the retina, allowing massive parallel processing of incoming visual information. It should be noted, however, that only in rare cases to RGCs or amacrine cells exhibit “perfect” tiling, in

which neighboring dendrites never overlap. Instead it seems that most cell classes show some overlap of processes. This fact will become important as I discuss the possible mechanisms of dendritic tiling.

Early studies performed in the retina provided evidence that tiling was established by repulsive interactions between dendrites (Perry & Linden, 1982). Depletion of RGCs in the developing retina caused the neighboring cells to grow towards the space previously occupied by the ablated RGCs. Similarly, tiling studies in da neurons have shown that repulsive dendrite-dendrite interactions organize class IV dendritic territories. Ablation of class IV neurons results in neighboring class IV neurons invading and arborizing the space once occupied by the ablated class IV neuron (Grueber et al, 2003b; Umera, 2003) . In addition duplication of class IV neurons lead to a complete split in the dendritic fields of the duplicated cells further suggesting that repulsion is necessary for limiting dendritic fields of class IV neurons. However upon duplication of class III neurons only secondary class III dendrite branches tile suggesting that tiling may only be necessary for refining terminal dendrite positioning of class III neurons. Duplicated class I- II neurons show extensive overlap suggesting that dendrite-dendrite repulsive interactions are not involved in establishing class I-II dendritic boundaries (Grueber et al, 2003b).

In *Brn3b (-/-)* mice, 80% of RGCs degenerate during development and the surviving RGCs fail to invade the vacant territory suggesting that tiling can also established without dendrite-dendrite contacts and other mechanisms must exist that determine dendritic field sizes (Lin et al, 2004).

So far we know very little about the molecular mechanisms that regulate tiling in da neurons. Studies in the fly visual system have been important for the identification of molecular mechanisms by which tiling can be regulated in other systems. Tiling of R8 photoreceptor axons requires heterophilic repulsive interactions between cell surface receptor *golden goal* (*gogo*) and an unknown ligand (Tomasi et al, 2008). As a result, *gogo* mutant R8 axons exhibit an axonal overlap phenotype only in the presence of adjacent R8 axons mutant for *gogo* (Tomasi et al, 2008). Down syndrome cell adhesion molecule-2 (Dscam2) regulates tiling of L1 lamina neurons through homophilic interactions (Millard & Zipursky, 2008). Single Dscam2 mutant axons fail to remain in their column and invade columnar territories of adjacent L1 neurons.

The above studies in the fly eye have been important for our understanding of different mechanisms that neurons can employ to set up their tiling borders. Studies aimed at understanding tiling behavior of da neurons have been carried out in class IV neurons. Mutant analysis of *flamingo* (*fmi*), a seven transmembrane cadherin, showed that it prevents overlap, as *fmi* mutant class IV neurons overgrow and exhibit dendritic overlap at the dorsal midline (Grueber et al, 2002; Kimura et al, 2006). Another set of molecules that were implicated in tiling includes Tricornered (Trc) and Furry (Fry) (Emoto et al, 2004; Han et al, 2012). Most dendrites of class IV da neurons are attached to the ECM ensuring that dendrites should be restricted to a 2D plane. Portions of dendrites are enclosed in the epidermis and removal of *trc* and *fry* causes excessive growth of dendrites into the epidermis and as a result the dendrites are no longer confined to a 2D plane. This

3D arrangement of arbors leads to overlap between dendrites present different planes (Han et al, 2012). Thus, tiling depends on 2D restriction of class IV arbors.

Presently very little is known about the cellular and molecular mechanisms by which classes of da neurons other than class IV neurons set up their territories. Dendrite-dendrite interactions, specific targeting cues and cell-substrate interactions are likely important for patterning of these dendritic fields. Understanding the cues that orchestrate the formation of dendritic territories of neurons will provide greater insights into the mechanisms employed in patterning of dendritic fields more generally, since strict tiling is an exceedingly rare phenomenon. In particular, the ability of class III neurons to respond and integrate signals from the environment and from adjacent neurons makes them an ideal model for dissecting the different growth inhibiting mechanisms that are important for shaping dendritic fields.

Regulation of shape and organization of dendritic fields by intrinsic mechanisms

Dendrites, like all other cells in an organism, do not develop in isolation. In addition to dendrite-dendrite interactions that establish and refine their neuronal fields, additional intrinsic mechanisms and extrinsic signals also play a role in the development of dendritic fields. Developing dendrites acquire their primary identity by the combinatorial action of transcription factors expressed by neurons (Jan & Jan, 2010; Parrish et al, 2007). In the *Drosophila* PNS, transcription factors (TF) determine class-specific branching patterns and the formation of dendritic territories. Studies in the past few years have

identified and characterized the role of several TFs in regulating dendritic patterning (Crozatier & Vincent, 2008; Grueber et al, 2003a; Kim et al, 2006).

Cut is a homeodomain TF that is expressed in a cell-type specific pattern in da neurons (Blochlinger et al, 1990; Grueber et al, 2003a). It has been shown to regulate dendrite growth, branching complexity and the size of dendritic territories (Grueber et al, 2003a). It is present at varying levels in different classes of da neurons except for class I neurons where it is absent. Cut is expressed at the highest level in the class III neurons followed by decreasing levels of expression in class II and IV neurons. Loss of Cut leads to a simplification of dendritic arbors and a reduction in the size of the dendritic field (Grueber et al, 2003a). Further, Cut over-expression caused dendrites of neurons to adopt actin-rich fillipodia seen in class III neurons and an expansion and extension of their dendritic fields. Thus, Cut levels correlate with an increase in branching complexity and expansion of dendritic territories. Conversely, the expression of a zinc finger TF Abrupt (Ab) in class I neurons has been implicated in restricting dendritic growth (Li et al, 2004; Sugimura et al, 2004). Removal of Ab from class I neurons leads their dendrites to adopt more complex branching patterns and ectopic expression of Ab in other classes of da neurons with more complex branching patterns results in a simplification of their dendritic pattern (Li et al, 2004; Sugimura et al, 2004). Similarly, another TF belonging to the Collier/ Olf-1/EBF family, Knot (Kn), also regulates dendrite growth and field size (Crozatier & Vincent, 2008; Hattori et al, 2007; Jinushi-Nakao et al, 2007). Kn is specifically expressed in class IV neurons. Similar to Cut, over-expression of Kn in other classes of da neurons induces a complex class IV-like branching pattern and more

extensive dendritic fields (Crozatier & Vincent, 2008; Jinushi-Nakao et al, 2007). In total, genetic analyses of Kn, Cut and Ab demonstrate the role of these TFs in control of class-specific size and complexity of dendritic fields.

The targets of these TFs are generally very poorly characterized. Studies have indicated that one of the ways Kn promotes dendritic branching and expansion of class IV dendritic territory is by regulating the transcription of a microtubule severing protein Spastin (Jinushi-Nakao et al, 2007; Roll-Mecak & Vale, 2005). Similarly, Cut mediates branching and formation of class III specific actin-rich fillipodia by regulating the transcription of actin bundling protein Fascin (Jinushi-Nakao et al, 2007). These studies suggest that the cell-type specific expression of TFs is critical for generating diverse dendritic field patterns and morphologies.

TFs also influence branching and size of dendritic arbors in vertebrate neurons by regulating growth-promoting factors, which in turn influence dendrite outgrowth and branching pattern (Schuurmans et al, 2004). Remarkably, the vertebrate homologs of Drosophila Cut, termed Cux1 and Cux2, have a very similar role in dendritic branch promotion as Cut does in da neurons (Cubelos et al, 2010). In addition, TFs also control the expression of microtubule associated proteins, growth factors and cell surface receptors that are needed to respond to signals in the environment and, which ultimately shape dendritic territories of neurons and enable dendrites to recognize neighboring cells.

Organization of dendritic fields by dendrite- substrate interactions

The role of guidance cues has been very well established in the targeting of axons and the formation of synaptic connections (Dickson, 2002; Tessier-Lavigne & Goodman, 1996). Classic guidance receptors such as the Robos, Semaphorins (Sema) and Frazzled (Fra) have been extensively studied for their roles in axon guidance in both invertebrates and vertebrates (Garbe & Bashaw, 2004; Kania & Jessell, 2003; Mitchell et al, 1996; Zlatic et al, 2003). However, only recently have their roles in targeted dendritic growth and field formation been uncovered. The regulation of dendritic fields by guidance molecules was first reported in apical dendrites of cortical pyramidal neurons, which were shown to be attracted to a source of Sema3A, indicating that guidance cues can control the orientation of dendrites (Polleux et al, 2000). Additional studies across different invertebrate and vertebrate model systems with different guidance cues have confirmed their general role in dendritic territory formation (Brierley et al, 2009; Furrer et al, 2003; Furrer et al, 2007; Godenschwege et al, 2002; Komiyama et al, 2007).

One example is Frazzled (Fra)/DCC, a cell surface receptor for the Netrin family of ligands. Classical studies showed that in response to Netrin, Fra promotes commissural axons to cross the midline (Kolodziej et al, 1996; Mitchell et al, 1996). In addition, the removal of Fra from a particular subset of neurons in the *Drosophila* CNS, the RP3 and the aCC neurons, results in dendritic branching and targeting defects, specifically lack of contralateral branch growth (Furrer et al, 2003). Likewise, removal Robo proteins (receptors for the Slit family of axon guidance cues) from aCC neurons also results in the loss of dendrites (Furrer et al, 2003; Furrer et al, 2007).

Insights into the role of guidance cues and their importance in dendritic field formation also come from studies carried out in the *Drosophila* olfactory system. Levels of Sema-1a are important for dendritic targeting of projection neurons to appropriate regions within the antennal lobe (Komiyama et al, 2007). Experiments where Sema-1a levels were altered cell autonomously in neurons caused a shift in their dendrites to regions in the antennal lobe that correlated with the levels in the projection neurons, i.e., higher levels of Sema-1a shifted the dendrites to higher-level antennal lobe regions (Komiyama et al, 2007).

Dendritic field sizes are also controlled by another mechanism known as scaling, which ensures that dendritic field sizes increase in proportion to the increase in animal body size (Parrish et al, 2009). Dendritic scaling of class III and IV neurons is regulated by signals from the epidermis. Studies have shown that *bantam*, a microRNA in the epidermis inhibits the phosphoinositide 3-kinase (PI3K) pathway in class III and IV neurons and as a result restricts dendritic growth (Parrish et al, 2009).

The role of morphogens has been well characterized in early development and patterning (Tabin, 1991). Originally morphogens were characterized as diffusible molecules that pattern fields of cells into molecular compartment that act as signaling centers that instruct tissue differentiation (Crick, 1970; Wolpert, 1969). However, studies in axon guidance have also implicated them as cues that regulate axon targeting and, more recently, in dendritic field establishment (Charron & Tessier-Lavigne, 2005). Morphogens such as Wingless (Wg) together with axon guidance cues play critical roles in axon guidance and synaptogenesis in invertebrates and vertebrates (Charron et al,

2003; Zou, 2004). Like in the case of commissural neurons in chicks where Netrin-1 acts together with Shh to guide the axons to the ventral midline(Charron et al, 2003; Salinas, 2003). Furthermore, Wnts have also been shown to regulate dendritic branching in the hippocampal neurons in mice and dendritic refinement of neurons in the *Drosophila* CNS (Ciani & Salinas, 2005; Osterfield et al, 2003). Wide field serotonergic neurons (CSDn) in the *Drosophila* CNS undergo remodeling during metamorphosis and the Wnt pathway is required for dendritic pruning to ensure that the dendrites only project to the antennal lobe. In the CSDn neurons the Wnt pathway is regulated in an activity dependent manner by the pre-synaptic neurons for CSDn neurons (Singh et al, 2010).

Morphogens and their diverse roles during development

Morphogens are essential for patterning of the *Drosophila* embryos as they are required for specifying dorso-ventral and anterior-posterior axes (Lawrence & Struhl, 1996; Zecca et al, 1995). Proteins belonging to the Wingless/Wnt, Hedgehog (Hh), and TGF-beta families have been identified as morphogens that regulate the formation of different body axes (Tabata & Takei, 2004). Initially, Engrailed (En) expression in a segmentally-repeated pattern defines posterior (P) compartments within each segment of the developing embryo (Blair, 1992; DiNardo et al, 1988). The compartmentalization of the segment and the border between them act as signaling centers which allow the cells of each compartment to respond differentially to signals (Basler & Struhl, 1994). En-expressing cells secrete Hh, which only the anterior (A) cells can respond to, which is followed by the activation of Wg signaling in the A cells (Moline et al, 1999). This sequential and precise activation of morphogens is indispensible to the development of

Drosophila embryo. The role of these morphogens has been well characterized during early developmental patterning, and now there is growing evidence that they are re-utilized during for later developmental processes including cell specification, migration, and neurite formation (Salie et al, 2005; Wilson & Stoeckli, 2012).

Hedgehog signal

Hedgehog (Hh) is present in both in insects and vertebrates. *Drosophila* has a single Hh protein and vertebrates have two homologs, Sonic Hedgehog (Shh) and Desert Hedgehog (Wilson & Stoeckli, 2012). No clear homolog exists in nematodes. Canonical Hedgehog signaling is primarily transduced via the interaction between two receptors, Patched (Ptc), a twelve-pass transmembrane protein (Ingham & McMahon, 2001; Saenz-Robles et al, 1995; Tabata & Kornberg, 1994) that binds the extrinsic Hedgehog ligand, and Smoothened (Smo), a transmembrane G-protein that ultimately transduces the Hh signal (Alcedo et al, 1996; Chen & Struhl, 1998; Ingham, 1989; Lawrence et al, 1999). In the absence of Hh, Ptc inhibits Smo activity, which leads to the inactivation of downstream Hh signaling components such as Cubitus interruptus (Ci) (Orenic et al, 1990). In the absence of the Hh signal, Ci is transcriptionally repressed and remains in the cytoplasm (Ingham & McMahon, 2001). To form a functional protein and transduce a signal, Hh must undergo cholesterol modification to yield an activated N-terminal domain. Once Hh binds to the Ptc receptor, it leads to subcellular localization and activation of Smo and the phosphorylation and cleavage of Ci/Gli is inhibited (Torroja et al, 2005). The active form of Ci translocates to the nucleus where it can activate the transcription of its target genes, which include *ptc* and *wg* among others. Shh regulates different development processes,

which include neurogenesis, hematopoiesis and limb development (Torroja et al, 2005; Wilson & Stoeckli, 2012). Furthermore, disruption of SHH is also related to multiple cancers and neurological disorders.

Although the Hh signal is generally propagated through the canonical Hh pathway for the majority of developmental processes it regulates, evidence is gathering for numerous non-canonical, Smo- and Gli-independent Hh signaling pathways (Jenkins, 2009). Studies in vertebrates have shown that the Shh signal is required for cytoskeletal arrangement and migration of fibroblasts (Bijlsma et al, 2008). However, when Gli is blocked using an antagonist it does not affect the chemoattraction induced by Shh required for the migration of the fibroblasts. Thus, in this system, the Shh signal can be transduced in a Gli-independent manner. In addition, there is also evidence of a Smo-independent transduction of Shh in the rostral migration of commissural axons in the developing spinal cord of chicks (Charron et al, 2003; Charron & Tessier-Lavigne, 2005), where removal of Smo by RNAi does not alter the trajectory of the migrating axons. Smo-independent transduction of the Hh pathway has also been supported by studies of the *Drosophila* olfactory neurons (ORN). Initial Hh signaling in the antennal lobe establishes two subsets of ORNs that express different levels of Ptc. The subset of ORN axons that express high levels of Ptc can respond to sources of Hh expressed later in development even though they do not express *smo* (Chou et al, 2010).

Additional non-canonical pathways are being uncovered outside of the nervous system. In *Drosophila* head development, Ptc and Smo act in concert to activate a receptor, Babo, an activin type I receptor to promote cell proliferation (Shyamala & Bhat, 2002). Thus,

evidence is building for different non-canonical ways for the Hh/Shh signal to be transduced and different ways in which the downstream signaling components interact. This diversity of signaling mechanisms may ultimately expand the roles for Hh/Shh in diverse developmental processes.

Roles of morphogens in the nervous system

The important role of morphogens in axon guidance, formation of synaptic connections and dendritic targeting is only starting to be appreciated. Studies have shown that Shh is required for axon guidance of commissural axons of the developing spinal cord (Charron et al, 2003; Charron & Tessier-Lavigne, 2005; Yam et al, 2012). Shh is secreted by ventral floor plate cells and acts as a chemoattractant, allowing the commissural axons to cross the midline (Placzek et al, 1990a; Placzek et al, 1990b). Once they cross the midline, the Shh signal is interpreted as chemorepellant by the commissural axons, which prevents them from re-crossing the midline. A second morphogen protein, Wnt/Wg also regulates axon guidance in the spinal cord (Yoshikawa et al, 2003). It is expressed along the longitudinal axis of the spinal chord and behaves as chemoattractant to guide commissural axons along the A-P axis. In addition, the Wnt pathway also regulates the guidance of mechanosensory neurons in *C. elegans* by repelling the axons anteriorly away from the Wnt source present in the posterior part of the embryo (Maro et al, 2009).

Very little is known about the role of morphogens in establishing dendritic fields. There is preliminary evidence that Shh in the dorsal-lateral portion of the midbrain is sufficient to maintain precise territories of neurons. The effect of the Wnt pathway on dendritic

branching and territory formation is slightly better understood (Ciani & Salinas, 2005). Wnts have been shown to control dendrite morphogenesis in hippocampal neurons in mice, where removal of Wnt or its receptor disheveled result in a decrease in dendrite branching and a reduction in dendritic length (Ciani & Salinas, 2005; Rosso et al, 2005).

Furthermore, Wnt/Wg have been implicating in establishing topographic maps in the optic tectum of the chick and the *Drosophila* visual system (Lyuksyutova et al, 2003; Sato et al, 2006). In the chick optic tectum, the ventral and dorsal RGCs respond differently to the Wnt signal, with the dorsal RGCs attracted to lower levels and repelled by higher levels, whereas the ventral RGCs are always repelled (Lyuksyutova et al, 2003; Rosso et al, 2005). Shh also regulates the formation of a specific cortical circuit in mice. Shh is expressed in layer V of cortical projection neurons and mice lacking functional Shh in the cortex form a smaller number of synapses and show abnormal dendritic growth (Harwell et al, 2012).

Morphogens together with guidance molecules are clearly important for wiring the nervous system (Raper & Mason, 2010). A recent study showed that Shh is required in commissural axons to respond to Sema mediated repulsion. In the absence of Ptc or Smo receptors the axons are unable to Sema signaling and exhibit gross guidance defects (Parra & Zou, 2010). As studies probe deeper into the formation of neuronal circuits the cross-talk between morphogens and guidance molecules in establishing neuronal connections has become more apparent. However, how morphogens and guidance molecules regulate dendrite morphology and restrict dendritic fields to specific regions within the body wall or CNS still remains an unanswered question.

Compartments and nervous system patterning

Compartments act as essential signaling centers that pattern neighboring cells (Perrimon, 1994). Similar to the division of the *Drosophila* larvae body wall into A and P compartments, the CNS too is also organized into A and P compartments (Doe & Technau, 1993; Garcia-Bellido et al, 1973; Lawrence & Struhl, 1996). In both flies and vertebrates neuronal branches have been found to be restricted within specific molecular boundaries (region defined by the expression of a morphogen or guidance cue) (Jessell et al, 2011; Landgraf et al, 2003). Anatomical studies in *Drosophila* have shown that motor neuron dendrites are restricted to specific compartments, such that dendrites of motor neurons that synapse with internal muscles are restricted to the P compartment and dendrites external muscles motor neurons are restricted to the A compartment (Landgraf et al, 2003).

P compartment cell identities are defined by Engrailed (En) a highly conserved homeodomain TF (Hama et al, 1990; Lawrence & Morata, 1976; Lawrence & Struhl, 1996; Tabata et al, 1995). En is very well studied for its role in patterning of the nervous system in vertebrates and invertebrates (Carpenter et al, 1993; Kornberg, 1981). En is shown to be important for imparting cell identity, axon guidance and cell survival. In mice, En is expressed in specific regions leading to the compartmentalization of the midbrain, hindbrain and spinal chord (Wurst et al, 1994). Removal of En from these parts of the brain results in the complete loss of cells and leads to premature death as they are

missing multiple cranial nerves (Hanks et al, 1995). In addition in the chick neural tube En defines the position of dorsal di-mesencephalic boundary by repressing the diencephalic fate (Araki & Nakamura, 1999). En also plays roles in axon guidance, it promotes the transcription of Ephrin A, which is expressed in a caudal (low) -to-rostral (high) gradient in the tectum, and repels retinal axons expressing high levels of EphA receptors causing them to project to the rostral tectum (low EphrinA region) (Cheng et al, 1995; Shigetani et al, 1997). Thus the En gradient via regulating the expression of EphrinA influences the guidance of retinal axons (Brunet et al, 2005). The most detailed analysis of the role of En in patterning the nervous system has been performed in the *Drosophila* CNS. The *en* gene is expressed in developing neuroblasts located at the posterior boundary of every CNS neuromere (Bossing et al, 1996; Younossi-Hartenstein et al, 1996). Neurons derived from these neuromeric lineages all project to identical neuropile compartments in the brain and furthermore their neuronal branches are restricted within those compartments (Kumar et al, 2009).

Functional consequences of specific dendritic territories

Neuronal form is likely to be intricately linked to function as it determines the synapses that can form; and the stimuli it can sample. However, only a few studies have been performed to show the importance of neuronal shape to its function.

In Drosophila, the da neurons are responsible for diverse sensory modalities. Class IV neurons receive nocioceptive and light responses (Kim et al, 2012b), class III neurons sense gentle touch, and class I neurons provide proprioceptive feedback (Hughes &

Thomas, 2007; Yan et al, 2013). It has been shown that the dendrite morphology of class IV neurons affects the ability of animal to respond to noxious stimuli. A microtubule severing protein Kat-60L1 regulates class IV dendrite morphology, *kat-60L1* mutants not only exhibit reduction in their branching complexity and length but also show deficits in sensing noxious stimuli (Stewart et al, 2012). However, much work remains to understand the underlying mechanisms of how arbor morphology and dendritic field size are linked to neuronal function

Tiling interactions and signaling cues from the substrate play important roles in determining axon and dendrite positioning and shape. They are likely to be important for ensuring proper territory coverage and circuit formation. This thesis addresses the role of tiling interactions and dendrite-substrate interactions in establishing dendritic boundaries and has identified novel mechanisms by which dendritic boundaries are defined, and how those borders may be important for neuronal function.

Figure 1.1 Distinct dendritic morphologies and territories of different classes of dendritic arborization (da) neurons (adapted from (Grueber et al, 2003b)

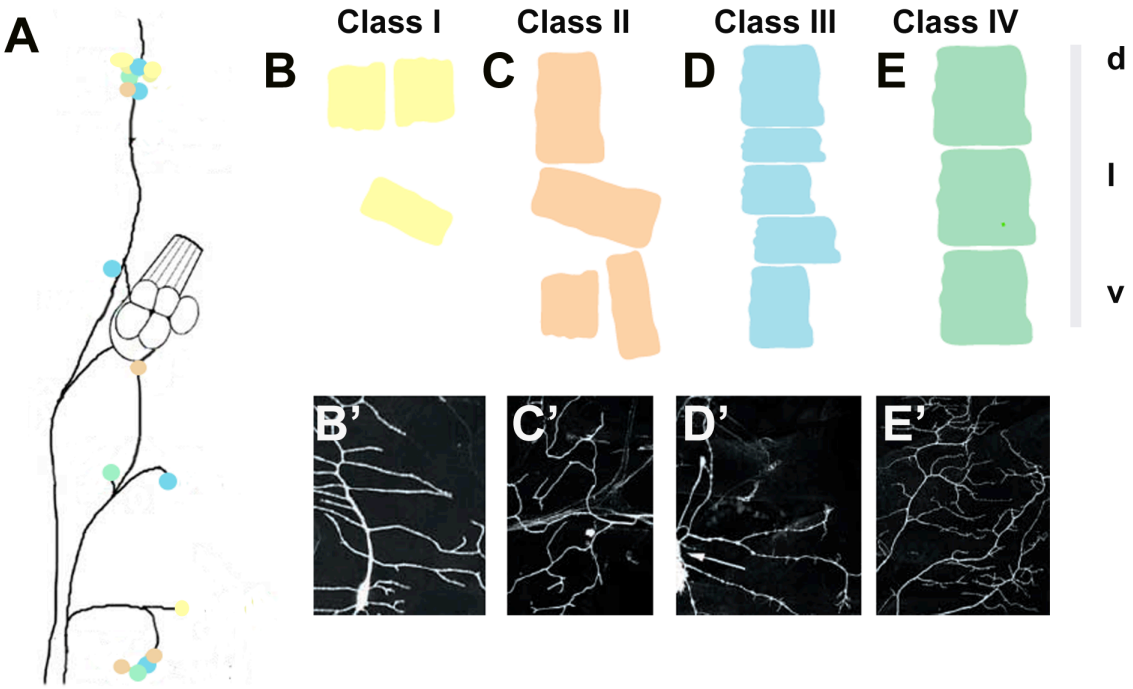


Figure 1.1 Distinct dendritic morphologies and territories of different classes of dendritic arborization (da) neurons

(A). Schematic showing the of arrangement of dendritic arbor neuron cell bodies. The different classes of neurons are color-coded. Class I- yellow, class II- tan, class III- blue and class IV- green.

(B-E). Schematics of the territories occupied by the different classes of da neurons in each hemisegment.

(B'-E'). Dendritic arbors of class I (B'), class II (C'), class III (D') and class IV (E') neurons (used from Grueber, 2002 with permission from Company of Biologists).

Figure 1.2 Self-avoidance and dendritic tiling in da neurons

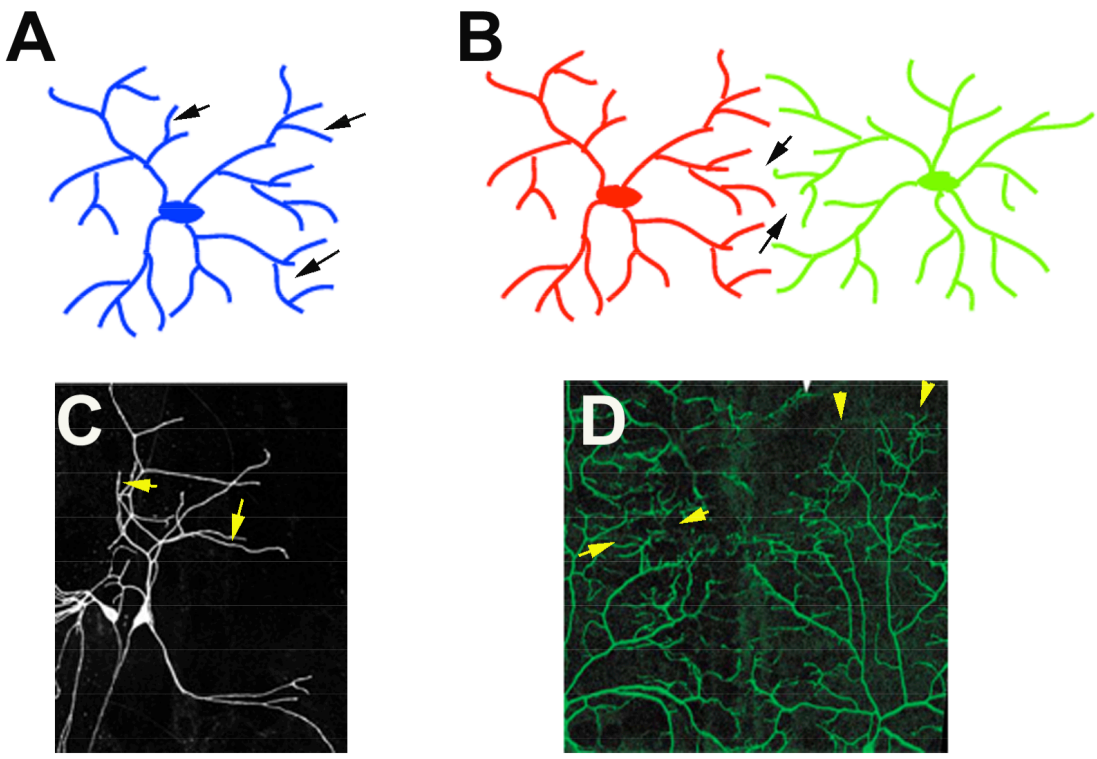


Figure 1.2 Self-avoidance and dendritic tiling in da neurons

(A). Schematic representing spacing between sister dendrites in a neuron. (arrows)

(B). Schematic representing tiling between adjacent dendrites in neighboring neuron. (arrows)

(C). A Dscam1 loss of function clone made using the mosaic analysis with a repressible cell marker (MARCM) system shows extensive overlap between sister dendrites (arrows) (used from Matthews, 2007 with permission from Elsevier Inc.).

(D). Class IV neighboring neurons invading the space formerly occupied by an ablated class IV neuron. Arrows represent the normal tiling borders in the contralateral segments. Arrowheads representing the invasion from adjacent segments (Grueber, 2003, permission).

Figure 1.3 Canonical Hedgehog signaling pathway

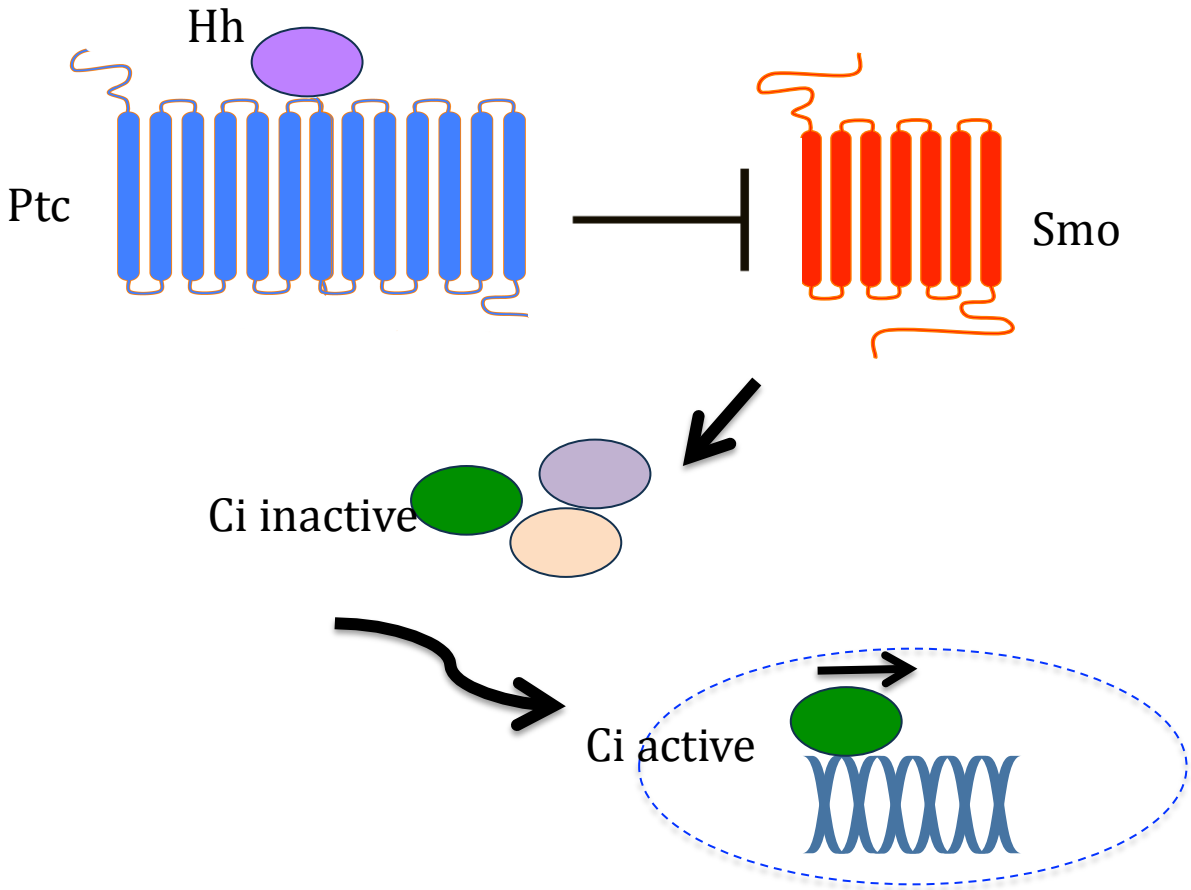


Figure 1.3 Canonical Hedgehog signaling pathway

A schematic of the canonical Hedgehog (Hh) pathway. Hh signaling is primarily transduced via the interaction between two receptors, Patched (Ptc), a twelve-pass transmembrane protein that binds the extrinsic Hedgehog ligand (blue circle), and Smoothened (Smo), a transmembrane G-protein that ultimately transduces the Hh signal. In the absence of Hh, Ptc inhibits Smo activity (grey repressor bar), which leads to the inactivation of downstream Hh signaling components such as Cubitus interruptus (green circle). In the absence of the Hh signal, Ci is transcriptionally repressed and remains in the cytoplasm (purple and yellow circles).

Methods

Fly stocks and genetics

General reagents

For visualizing and manipulating da neurons I used *spineless GAL4, UAS mCD8:: GFP* (Jenett et al, 2012) and *189Y GAL4, UAS mCD8:: GFP* (Matthews & Grueber, 2011) for class III neurons, *221 GAL4, UAS mCD8:: GFP* (Grueber et al, 2003b)for class I neurons and *109(2) 80 GAL4, UAS mCD8:: GFP* (Gao et al, 1999)for all da neuron classes.

Generation of FLP-out clones

Mosaic FLP-out clones for analysis of wild-type neurons or for overexpression experiments were generated as previously described by crossing males from control (*w1118*) or lines carrying various UAS overexpression constructs to females of the genotype *hsFLP; 109(2)80 Gal4, en-lacZ; UAS>CD2, y+>mCD8::GFP* (Grueber et al, 2007) and delivering a 30-45 mins heat shock to first instar larvae approximately one day after egg laying. For FLP-out experiments examining da neuron dendrite tiling and boundaries *hsFLP; 109(2)80 Gal4, en-lacZ; UAS>CD2, y+>mCD8::GFP* virgins were crossed to *ppk-eGFP* males. To study the effects of ci^{R} and ci^{Act} over-expression on class I neurons, *hsFLP; 109(2)80 Gal4, en-lacZ; UAS>CD2, y+>mCD8::GFP* females were crossed to males of *UAS-* ci^{R} and *UAS-* ci^{Act}.

MARCM clones

MARCM clones were generated as previously described using *hsFLP, C155-Gal4, UAS-mCD8::GFP; FRT 42D, tubP-Gal80/CyO* crossed to the appropriate mutant allele on a *FRT 42D* and *FRT 40A* chromosome. Heatshocks in a water-bath were provided approximately 4 hours after egg laying for 2x 30 minutes, with recovery at room temperature for 30 minutes in between. Wild-type control clones were generated using *FRT42D, w+* or *FRT 40A, w+*. Third instar larvae with clones were dissected and stained as described below.

Analysis of embryonic mutant phenotypes

To identify potential receptors that restrict class III dendrites to the A compartment candidate mutant alleles were balanced with a marked GFP balancer and combined with a class III neuron specific Gal4 driver, *ss-Gal4, UAS mCD8 :: GFP*. To examine the effects of the mutants, 12-16 hr embryos were gently bleached for 1 min, mounted on a slide with 70% glycerol and examined live by confocal microscopy.

Cell ablations

To ablate class III neurons, *spineless GAL4* (Rubin line # 20310) a class III specific driver was used. *UAS mCD8:: GFP* was recombined to *spineless GAL4* to visualize class III neurons. Ablations were performed in stage 12-16 embryos grown at 25°C. The embryos were dechorionated in 100% bleach for approximately 2 minutes. The embryos were then arranged at the edge of a grape plate with the dorsal side of embryos facing up. To make sure the embryos did not dry a humidifier was used. The embryos were then

transferred to a slide using double -sided tape, which ensured that the embryos did not move during ablation. The embryos were covered with a mixture of equal volumes of series 27 and 700 halocarbon oil. The embryos were covered with a cover slip prior to ablation. The ablations were performed using a Micropoint dye pulsed laser (Photonic Instruments). The laser targeted at the nucleus of the cell and the strength of the laser was set such that 1-2 pulses were sufficient to kill the cell. Embryos were allowed to recover at 18°C in large petri dishes containing series 27 halocarbon oil. Once the embryos hatched they were transferred to a vial using a thin paint -brush. The vial was transferred to 25°C and the larvae were allowed to develop to the third instar stage and dissected.

Data was analyzed using the methods described in (Grueber et al, 2003b)

Engrailed misexpression

Engrailed (En) is normally expressed in the P compartment. To test whether En is sufficient to restrict class III dendrites I misexpressed *en* in A compartment cells. I crossed *hsFlp; Act5c<CD2< Gal4* to homozygous *UAS-en; UAS CD8 GFP*. Embryos were heat-shocked for 1 hour at 37°C at the end of a 2 hour collection. The embryos were then aged at 25°C, allowed to develop to the third instar, and dissected. The dissected larvae were labeled with anti-GFP and anti-HRP antibodies. GFP positive cells were scored as en misexpressing cells.

To quantify whether class III dendrites avoided en expressing A compartment epidermal cells I counted the number of class III dendrites that elaborated over en expressing cells and divided this result by the area of the cell. The result gave me the crossings/area. For

the control the same quantification method was used for non-GFP cells in the A compartment of adjacent segment.

Analysis of robo3 mutants

To examine the compartment preference of vdaD dendrites in robo3 mutants I crossed *robo3^{3}/cyo$_{nucGFP}$; hh-lacZ* flies to *robo3^{3}/cyo$_{nucGFP}$; spineless ^{GAL4}, UAS mCD8:: GFP* flies. Progeny that lacked nuclear GFP expression were dissected and stained with GFP, HRP and β-Gal antibodies. To quantify whether vdaD dendrites grow into the P compartment in *robo3* whole mutants; I traced class III dendrites in the control and *robo3* whole mutants that grew over P compartment cells. The P compartment was marked using *hh-LacZ*, a P compartment reporter.

Fly stocks: Chapter 2

To visualize and ablate class III neurons I used *spineless GAL4, UAS mCD8:: GFP* (Rubin Collection). I used *hsFlp; 109(2)80 Gal4, en-lacZ, UAS <CD2<CD8 GFP* FLP-out constructs to mark individual da neurons and the P compartment. To perform genetic ablation I used *189Y; ato^{1}*, which allowed me to visualize class III neurons in an *ato^{1}* mutant background.

Fly stocks: Chapter 3

The following fly stocks were used and generated to screen potential molecules that play role in restricting class III dendrites to the A compartment 1. *robo^{3}; ss-Gal4, UAS mCD8 GFP*, 2. *robo 1,2,3; ss-Gal4, UAS mCD8 GFP*, 3. *sli^{2}; ss-Gal4, UAS mCD8 GFP*, 4. *netAB;*

ss-Gal4, UAS mCD8 GFP, 5. *netAB; robo*^{3}; *ss-Gal4, UAS mCD8 GFP*, 6. sema-1a^{KY}; *ss-Gal4, UAS mCD8 GFP*, 7. *plex B*^{KYG40}*; ss-Gal4, UAS mCD8 GFP*, 8. *wg*^{X4}*; ss-Gal4, UAS mCD8 GFP*, 9. *en*^{1}*; ss-Gal4, UAS mCD8 GFP*, 10. *hh*^{21}*; ss-Gal4, UAS mCD8 GFP*, 11. *ptc* ^{IIW}*; ss-Gal4, UAS mCD8 GFP* and 12. *eph*^{x652}*; ss-Gal4, UAS mCD8 GFP*.

To generate *robo3* clones and label the P compartment *FRT40A, robo3*^{3}*;hh*^{P30} was used. To ectopically express en in the A compartment *hsFlp; Act5c<CD2<Gal4* virgins were crossed with *UAS-en; UAS mCD8 GFP* males.

I crossed the following UAS lines with *hsFLP, 109(2)80 Gal4, en-lacZ; UAS <CD2<CD8 GFP* flies to screen for cell surface receptors that are sufficient to restrict class IV dendrites to the A compartment. *UAS- robo3*, *UAS- robo2, UAS- robo*, *UAS- unc-5*, *UAS- ephrin*, *UAS-sema 1a, UAS- plexB*.

To examine whether Hh regulates class I dendritic fields I crossed *wg- Gal4* to *UAS-hh*. To further whether the change was a result of the shift in the primary dendrite or a result of change in the receptive field, *en-lacZ* was used to mark the P compartment. I used the following fly lines to examine cell autonomous effects of the Hh signaling in class I neurons. I used the MARCM technique (Abuqamar et al, 2008) to perform these experiments all the mutant FRT lines on chromosome 2R were crossed to *hsFLP, C155-Gal4, UAS-mCD8::GFP; FRT 42D, tubP-Gal80/CyO* and the mutant FRT lines on the 2L arm of chromosome 2 were crossed to *hsFLP, C155-Gal4, UAS-mCD8::GFP; FRT 40A, tubP-Gal80/CyO*. The mutant lines used are as follows, 1. *FRT 40A, smo*^{3}, 2. *FRT 42D, ptc*^{S2}, *FRT 42D, ptc*^{IIW}, 3. *FRT 42D, ptc*^{IIW}*, en-lacZ* and the control used were 4. *FRT42D, w*$^{+}$ and

5. *FRT40A, w+*. To investigate whether Ci plays a role in regulating primary dendrite position I overexpressed both repressor and activated forms of Ci, *UAS-ci^{R}* and *UAS- ci^{Act}* by crossing to *hsFLP, 109(2)80 Gal4, en-lacZ; UAS <CD2<CD8 GFP*.

Fly stocks: Chapter 4

For live imaging studies *NompC-QF, QUAS-tdTom; 221 Gal4, UAS mCD8 :: GFP* flies were crossed to *w+* flies. To perform behavioral analysis *221 Gal4* and *tsh- Gal80; 221 Gal4* flies were crossed to *UAS-tnt* and *UAS-tnt ^{inactive}*. To examine whether a shift in vpda primary dendrite position causes crawling behavior defects *wg-Gal4* was crossed to *UAS-en* and to *w+* as controls.

Larval dissection and immunohistochemistry

Third instar larvae were first pinned at anterior and posterior ends in Sylgar dishes filled with 1X PBS and cut through the dorsal midline. Four additional pins were used to flatten the larva prep by pining down the dorsal edges of the larva, in a fillet preparation. 4-6 larvae were dissected in a single dish, with no more than 15 minutes elapsed from the first to the last. Preparations were then fixed in 4% paraformaldehyde (Electron Microscopy Sciences) in PBS for 15 minutes and transferred to 5 ml rounded tubes, and rinsed for 3x5 minutes in PBS + 0.3% Triton X-100 (PBS-TX). Pre-block was performed for 1 hr at 4°C with 5% normal donkey serum (Jackson Immunoresearch, West Grove, PA) prior to the addition of

appropriate primary antibodies diluted in 200 microliters of PBS-TX (concentrations listed below). Primary antibody incubation proceeded for approximately 24-48 hours at 4°C or for 8-12 hours at 25°C. Rinses were performed in PBS-TX , followed by incubation in secondary antibodies diluted in 200 microliters of PBS-TX and incubation for 24-48 hours at 4°C or 8-12 hours at 25°C. Larvae fillets were mounted on poly-L-lysine coated coverslips and dehydrated in ethanol series (30%, 50%, 70%, 95%, 100% I, 100% II), each for 5 minutes. Tissue was cleared in xylenes (2 x 10 minutes), and mounted in DPX mounting medium (Fluka, Sigma-Aldrich). For a detailed protocol and discussion of methodology, see the following references: (Grueber et al., 2002; Shrestha and Grueber, 2010).

Antibodies

The following primary antibodies were used:

• goat anti-HRP (1:250, Jackson Immunoresearch)

• chicken anti-GFP (1:1000-1:2000, Abcam, Cambridge, MA)

• mouse anti-GFP (1:250, Molecular Probes/Invitrogen, Carlsbad, CA)

• mouse anti-22C10 (1:100, developed by S. Benzer and distributed by Developmental studies Hybridoma Bank (DSHB), Iowa City, IA)

• mouse anti-CD2 (1:250, abD Serotec)

• rat anti-CD8 (1:100, Cappel, now a part MP Biochemicals)

• rat anti-Cadherin (1:10, Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA) Rhodamine Red-X, FITC, Cy2 and Cy5-conjugated secondary antibodies were used against the appropriate species (1:200; Jackson Immunoresearch).

Image acquisition and analysis

Images were acquired on a Zeiss 510 Meta confocal microscope using 40X Plan Neofluar 1.3 N.A objective. Custom settings and appropriate lasers were used to acquire the images. Z-stacks were projected to produce a single image using the Zeiss LSM software and Zen software. Confocal images were processed in Photoshop CS3 (Adobe Systems) or Zeiss LSM software (Carl Zeiss, Germany). Dendrites were traced or marked using Neurolucida (MBF Bioscience). Quantification was performed with Neurolucida Explorer (MBF Bioscience), custom and with ImageJ. Quantifying dendrites using modified Sholl analysis Photoshop CS3 was used. Statistical analysis was performed in R (R Development Core) and Excel (Microsoft).

Live imaging

To examine class I and class III dendrites during larval crawling live imaging using a multi-photon excitation microscope (Nikon A1R MP system, Nikon Corporation, Japan) was used. The animals were placed on ice to slow their crawling and then mounted in 50:50 mixture halocarbon oils series 27 and 440. The crawling behavior of the larvae was then recorded for approximately 2 min using the NIS-elements microscope imaging software (Nikon software). The video files were edited using ImageJ.

Modified Sholl Analysis

To quantify changes in dendrite polarity we devised a quantitative method that we termed asymmetry index (a.i.). The primary dendrite was traced using Photoshop CS3. This tracing was placed at10μm distances from the primary dendrite across the entire dendritic field anterior and posterior to the primary dendrite was covered. Dendrite crossings at each of the10μm tracings were recorded. The sum of dendrite crossing was computed and the ratio of the number of dendrite crossings at each of the 10μm distance to the total number of dendrites was calculated. If the dendrites were anterior to the primary dendrite, the ratio was considered as positive and negative for dendrite that lay posterior to the primary dendrite. All the ratios were summed up to give the final number, which we termed as the a.i. A positive a.i. represents anterior polarization of class I secondary dendrites, a negative value represents posterior polarization of secondary dendrites, and a value of 0 indicates symmetry of the secondary dendrites.

Behavioral analysis

For crawling behavior analysis *221 Gal4* and *tsh-Gal80; 221 Gal4* were crossed to *UAS-tnt* or w^{1118} or $UAS\text{-}tnt^{inactive}$. The crosses were kept at the optimal temperature of 25°C and passed to fresh food everyday to ensure the larvae being examined from a vial were of similar ages. After approximately 5 days, larvae were removed and placed on a petri dish and cleaned in 1X PBS. The cleaned larvae were placed on 1% Agar plates and given 2 minutes to acclimatize, after which their crawling behavior was recorded for 63 seconds using the Multi Worm Tracker (MWT) software program and crawling speeds were

analyzed using a customized software Eclipse (developed by Caline Karim in the Javitch lab).

Statistics

Statistical tests were performed using R and the data was presented in the form of boxplots. Thick lines represent the median, boxes represent second and third quartiles and data points that lie outside this range are represented as open circles. Bar graphs, when used, represent mean ± standard deviation. To check whether the data set was normal the Shapiro-Wilk normality test was performed. All p values represented as: *=$p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$. Significance for normally distributed data was assessed by performing a Student's t-test.

Chapter2

Cellular Basis of Tiling

Introduction

Dendritic fields are important determinants of neuronal function as they influence the receptive fields of sensory neurons and are critical for forming connections with the appropriate synaptic partners, thus ensuring appropriate sampling of sensory and synaptic information (Grueber & Sagasti, 2010).

Many organizing principles contribute to the patterning of dendritic fields. First, dendrites have the ability to discriminate self from non-self and as a consequence branches belonging to same neuron repel each other. This phenomenon is termed self-avoidance, and ensures that dendritic branches of a neuron are evenly spread over a territory (Kramer & Kuwada, 1983). Second, neurons belonging to the same class have unique dendritic fields that do not overlap, known as tiling (Grueber et al, 2003b). Tiling among neurons prevents overlapping coverage of the space. Third, unlike exclusive territories that exist between homotypic neurons, dendritic fields of neurons from different classes overlap extensively, and thus co-exist. Thus repulsive interactions among sister dendrites and interactions between neighboring neurons determine the final shape and extend of dendritic fields (Blackshaw & Thompson, 1988).

To understand the mechanisms underlying dendritic boundary formation, we examined dendritic arborization (da) neurons in the *Drosophila* peripheral nervous system. da neuron dendrites spread across epidermal cells and at the boundaries of their fields engage in dendrite- substrate/ dendro-dendritic interactions which could possibly halt

arbor growth. da neurons are categorized into four morphologically distinct classes (Class I- IV in order of increasing branching complexity(Grueber et al, 2002). In addition to possessing distinct morphologies, each class of da neurons stereotypic dendritic fields (Grueber et al, 2002). Class I-II neurons have small restricted fields, class III dendrites have larger fields, which together cover ~70% of the epidermis, and dendrites of class IV neurons cover the entire epidermis in a non-redundant manner.

Cellular and molecular mechanisms that enable neurons to discriminate self from non-self have been elucidated both in invertebrates and vertebrates (Lefebvre et al, 2012; Matthews et al, 2007). However very little is known about tiling interactions that occur among dendrites of neighboring neurons. So far only the nature of cellular mechanisms that determine tiling borders of class IV neurons has been uncovered. Repulsion between class IV dendrites was demonstrated by performing two types of experiments - ablation and over production of class IV neurons (Grueber et al, 2003b). Ablation experiments performed in class IV neurons showed that repulsive interactions between dendrites of neighboring homotypic neurons are responsible for sculpting the dendritic fields of class IV neurons. Next, duplicated class IV neurons were examined in *hamlet* (*ham*) mutants. In *ham* animals external sensory (es) neurons adopt a multidendritic neuron fate, occasionally leading to the doubling of the class IV neuron v'ada (Grueber et al, 2003b; Moore et al, 2002). Duplication of v'ada leads to the partitioning of the dendritic fields of the duplicated neurons such that their dendrites occupy distinct non-overlapping parts of the epidermis. However, duplication studies in class III neurons led to overlap among the primary dendrites of

the duplicated neurons and tiling only occurred between their tertiary branches. This result suggested that repulsive interactions among class III dendrites may refine dendritic tiling borders but are unlikely to be central to class III tiling (Grueber et al, 2003a). Furthermore, anatomical studies examining the dendritic fields of da neuron have shown their dendritic fields may not be continuous and spaces can occur in their coverage of the epidermis (Grueber et al, 2002). Therefore, other mechanisms in addition to homotypic repulsive interactions must exist that regulate the formation of dendritic fields of neurons. Additional mechanisms such as dendrite-dendrite repulsive interactions between neurons that belong to different functional classes or cues from the substrate their dendrites innervate may be utilized by neurons as alternative ways to establish their dendritic boundaries.

In this chapter I performed experiments to investigate dendritic tiling interactions that occur between homotypic and heterotypic neurons. The results from these experiments will help me identify the potential repulsive dendrite –dendrite interactions that occur between neurons and how they influence dendritic boundaries.

Results

Role of homotypic repulsion in class III dendritic tiling

Duplication of class III neurons provided preliminary evidence that class III tiling borders maybe refined by repulsive interactions that occur between neighboring class III dendrites (Grueber et al, 2003b). However, ablation studies have not yet been performed to demonstrate whether repulsive interactions are required for establishing

class III tiling borders. I wanted to investigate whether like in the case of class IV neurons, repulsive dendrite-dendrite interactions played a role in forming class III tiling boundaries. In order to address this question I ablated vdaD neurons in embryos and assessed whether there was change in the dendritic length of the adjacent class III neuron v'pda in third instar larvae. The class III GAL4 driver *spineless* (*ss)-GAL4* was used to visualize the vdaD neurons in embryos for ablation using a dye-pulsed laser (Figure 2.3 A). I quantified the results by measuring the maximum dendrite extension of v'pda towards the ablated vdaD and compared it to the maximum dendrite extension of v'pda in the adjacent segment where vdaD was present (Grueber et al, 2003b). The resulting ratios were compared to the control ratios and no significant differences were observed (p-value 0.3, Figure 2.3 B and C).

In order to ensure that the lack of growth by v'pda dendrites towards the space previously occupied by vdaD was not due to damage to the epidermis, I performed additional ablations in embryos where the epidermis was labeled with an epidermal marker E-cadherin (E-cad). There were no obvious differences in E-cad staining between ablated and unablated segments, which confirmed that lack of growth of v'pda dendrites into territory of the ablated vdaD neuron was not as a result of damage to the epidermis during the course of ablation. Although the change in the growth of v'pda dendrites in hemi-segments lacking vdaD was not significant when compared to control segments, occasionally a single v'pda dendrite extended into the space once occupied by the ablated cells, suggesting that homotypic repulsive interactions between dendrites of neighboring class III neurons may play role in refining class III

dendritic boundaries rather than the being instructive for the formation of class III tiling boundaries like in the case of class IV neurons.

Tiling boundaries exist between different classes of da neurons

As homotypic repulsive interactions between the dendrites of adjacent class III neurons did not appear to play a major role in restricting class III dendritic borders, we wanted to investigate whether class III neurons formed tiling borders with dendrites of other classes of da neurons. In order to assess whether tiling existed between different classes of da neurons we quantified the number of overlaps between the dendrites among different pair of da neurons, where each neuron in the pair belonged to a different class. Quantifying overlaps is one method of assessing whether dendrites of adjacent heterotypic neurons tile, if their dendrites overlap it would suggest that they can co-exist and on the contrary if there is no dendritic overlap between the neurons it would suggest that they tile (Fig. 2.1 B,C).

We focused our analysis on class I-III neurons in the ventral cluster. On quantifying the dendritic overlaps between different pairs of ventral cluster neurons, we found that the dendrites of class I neuron vpda and class III vdaD tile as their dendrites showed minimal to no overlaps in the six pair that were quantified. Similarly, no dendritic overlaps were seen between class II neuron vdaC and class I vpda. However, we found there to be extensive overlap between the dendrites of class II vdaC and class III vdaD.

These data suggest that tiling can occur between heterotypic neurons and as a result heterotypic pairs occupy distinct territories. Repulsive interactions between

heterotypic pairs could provide an explanation for these territory restrictions. In addition these results indicate that tiling is not limited to homotypic neurons and heterotypic tiling interactions may be used as a mechanism to ensure that neurons belonging different functional classes innervate specific regions within the body wall.

Dendritic boundaries are maintained throughout development and are not a consequence of late stage remodeling

The dendritic boundaries I observed in vdaD neurons could set up in multiple ways. Dendritic boundaries of vdaD may either be established during initial dendrite patterning or as a result of late stage remodeling. To examine whether the dendritic boundary of vdaD is established de novo or as a result of later remodeling, I visualized the dendritic boundary of vdaD neurons in embryos and the different larval stages using spineless (*ss)- GAL4, UAS mCD8::GFP*, which specifically labels the dendrites of class III neurons in the ventral cluster. In order to distinguish between these two possibilities and inability to label the epidermis in live animals, I examined the space between the dendritic fields of the vdaD neurons in adjacent segments. If the vdaD dendritic boundary is established early during development the space between the dendritic fields of vdaD neurons in adjacent segments should be present early in development and persist unchanged to later stages. On the contrary, if vdaD dendritic boundaries are formed as a consequence of late stage remodeling, either tiling or overlap between dendrites of vdaD neurons present in adjacent segments might be

observed during development, and then be pruned by late larval stages. On examining the dendritic boundaries of vdaD during different stages of embryonic and larval development, I found that a characteristic space between the dendrites of vdaD neurons in adjacent segments was present during early embryonic stages and maintained throughout development (Fig. 2.2). This result suggests that vdaD dendritic boundaries are established during early embryonic stages and are unlikely to be formed as result of late stage remodeling. vdaD neurons do increase in complexity by addition of tertiary branches during development, but this increase in branching does not change the dendritic boundary.

Tiling is not established primarily by heterotypic interactions

In the ventral cluster, posterior dendrites of vdaD neurons form a tiling boundary with anterior dendrites of vpda. In order to investigate whether the tiling boundary was established by repulsive heterotypic interactions between vdaD and vpda, I genetically ablated the class I neuron (Fig. 2.4A). To investigate heterotypic interactions I studied *atonal* (*ato*) mutants as ventral class I neurons (vpda) fail to be specified in these animals (Jarman et al, 1993). So at no point during development can vpda signal to the dendrites of vdaD. In *ato* mutants, class III dendrites were visualized using the class III specific GAL4 driver, *189Y Gal4, UAS mCD8 :: GFP* (Figure 2.4 B and C). To determine the role of vpda in establishing vdaD tiling border I quantified the distance of dendritic branch extension toward vpda starting from the point that dendrites become attached to the epidermis (see materials and methods for details). The space that is normally occupied by vpda was still void of class III dendrites in *ato* mutant

larvae. These data suggest that repulsive interactions between class I and class III neurons are not responsible for tiling.

Given that I did not observe major dendritic remodeling of vdaD when vpda was missing, we next performed a modified Sholl analysis to assess whether any extra growth of vdaD dendrites could be detected in *ato* mutants. Indeed, we found that very distal dendrites of class III neurons showed a significant increase in their branching density in *ato* larvae when compared with wildtype animals (Figure 2.4D and F). These results suggest that repulsive interactions occur between class III and I neurons, but are not solely responsible for the complementary, non-overlapping fields innervated by these cells.

Next, I quantified the dendritic field of the vpda neuron when the vdaD neuron was ablated. Reasoning that if a repulsive force is removed upon ablation of vdaD I would observe more extensive anterior dendrite growth in vpda, I used the asymmetery index (a.i.) to measure the dendrite extension of class I dendrites into the space formerly occupied by vdaD neuron (for details refer to materials and methods). Notably, according to this analysis class I neurons did not a show a change in the relative proportion of anterior and posterior dendritic extension (Figure 2.4F). Together from these results we conclude that repulsive dendrite-dendrite interaction between vdaD and vpda are not solely responsible for establishing the tiling border between the two neuron types.

Class specific patterns of da sensory neuron dendritic territories

Alternatively, molecular cues from the epidermis may influence territories of different classes of neurons. The epidermis of *Drosophila* as is divided into segments, where each segment is divided into an anterior (A) and posterior (P) compartment (Lawrence & Struhl, 1996). The posterior compartment is defined by the expression of Engrailed (En) and Hedgehog (Hh), whereas the posterior part of the A compartment expresses Hh receptor Patched (Ptc) and the morphogen Wingless (Wg). We screened a battery of epidermal markers to investigate whether dendritic boundaries of different classes of neurons correlated with the molecular boundaries in the epidermis. Individual neurons were visualized in animals where the P-compartment was labeled with *en-lacZ*. The dendrites of the class I neuron (vpda) are primarily restricted to the P compartment (Fig. 2.5 C), and the primary dendrite grows along the A-P compartment boundary and shorter anterior dendrites arborize in the *ptc* domain in the A compartment. In contrast, the dendrites of class II/III neurons are restricted the A compartment and preferentially arborize in the *ptc* and *wg* compartment (Fig. 2.5 A,B). Unlike the class I-III neurons that exhibit compartment preferences class IV neurons show no bias and arborize both the A and P compartment. Two additional epidermal markers, *decapentaplegic* (*dpp*) and *sloppy paired 1* (*slp1*), showed no obvious correlation with dendritic boundaries of da neurons. Thus, different neuron classes display specific compartment preferences and our results show that dendritic boundaries correlate with molecular boundaries in the epidermis (Fig 2.5D).

Discussion

Dendritic territories are essential for appropriate sampling of the receptive field and are likely critical for processing sensory stimuli (Grueber et al, 2003a), Sagasti, 2005, Sugimara 2003}. Previous studies have shown that repulsive interactions between morphologically identical neurons can determine the extent of dendritic fields in a subset of RGCs. (Blackshaw 1982, Wassle 1985, Grueber, 2003, Oyster 1995 Masland). It is thought that such interactions are functionally significant since they ensure neurons of the same functional type cover a receptive area completely and non-redundantly. The major finding of this chapter extends the conventional view of tiling to heterotypic neuronal types. This finding raises questions about the cellular, molecular, and functional relevance of this new category of tiling, which I address in turn below.

Cellular basis of heterotypic tiling

Class III neurons innervate approximately 70% of the epidermis but show characteristic gaps in their dendritic fields (Grueber et al, 2002). Thus unlike class IV neurons their dendritic fields cannot be explained entirely by tiling. The results from my class III vdaD ablation studies suggest that class III neurons are likely to require additional growth inhibiting for setting up their tiling boundaries. These additional dendritic inhibiting cues may come from neighboring heterotypic neurons or from the epidermis (addressed below). To support this idea, our analysis of dendritic overlaps

suggested that tiling can occur between dendrites of different neuron pairs in the ventral cluster.

Prior studies have shown that dendritic fields of neurons with different sensory modalities overlap extensively and remain largely unaffected by neurons of different functional or morphological classes (Blackshaw et al, 1982; Lin et al, 2004; Masland, 2001; Vaney et al, 1991). Ablation studies conducted on three classes of functionally distinct neurons in the leech demonstrated that tiling occurs between functionally identical cells, such that if a neuron of a specific modality was ablated, only adjacent neurons that belonged to the same sensory modality invaded the vacant receptive field (Blackshaw et al, 1982).

However, our quantification of dendritic overlaps between different pairs of da neurons in the ventral cluster we found that morphologically and functionally distinct neurons form tiling borders. In particular, class I-III neuron pair, vdaD and vpda formed a tiling border. On the basis of behavioral assays and the location of their axon projections it has been suggested that Class I neurons transduce proprioceptive information (Grueber et al, 2007) (Hughes & Thomas, 2007). More recently, class III neurons were shown to sense subtle mechanical stimuli termed gentle touch (Han et al, 2012). The results of this analysis lead us to test whether neurons of distinct sensory modalities may have the ability to identify and regulate dendritic boundaries of their heterotypic neighboring neuron, in the same way as homotypic class IV neurons do (Grueber et al, 2002; Grueber et al, 2003a). We hypothesized that repulsive interactions between the two neurons may result in exclusive dendritic fields. We

tested this idea by genetically ablating the class I neuron. In the absence of vpda the dendritic boundary of vdaD remained unaffected. Likewise, ablation of vdaD did not result in the growth of vpda into the vacant space. This lack of change in the tiling boundary could conceivably be due to three reasons; repulsive interactions do not play role in establishing the tiling boundary between class I-III neuron pair, they act in a redundant manner with additional cues, or heterotypic interactions maybe important for establishing tiling boundaries, but not maintaining them.

Molecular basis of heterotypic tiling

Anatomical observations suggested that extrinsic cues present in the epidermis may contribute to the formation of heterotypic tiling borders. Indeed, da neurons in the ventral cluster are organized in a compartment specific manner such that their dendritic boundaries correspond to the compartment boundaries in the epidermis (Lei, Y, Lee, J and Grueber, W. unpublished results).

In addition, compartment specific organization of motor neuron dendrites has been reported in the *Drosophila* CNS, the CNS like the epidermis is divided into alternating compartments (Landgraf et al, 2003). Furthermore, in the CNS the dendritic fields of a pair of motor neurons formed tiling borders, where their respective dendritic fields were restricted to specific compartments.

It is possible that molecular mechanisms that specify compartment identity in the epidermis also limit the dendritic boundaries of neurons that are located within

specific compartments independent of dendritic interactions among heterotypic tiling neurons.

So far, it remains unclear whether molecular boundaries in the substrate can regulate tiling boundaries of neurons. In order to address this question, I studied the role these compartment cues, specifically the role of *hh* and its signaling components in determining dendritic boundaries of vpda in chapter 3, as the boundary of Hh correlated with the dendritic territory of vpda. In addition, I also investigated the potential role of *en* (P- compartment) in restricting class III dendrites to the A compartment. Dissecting the roles of these boundary cues in determining dendritic territories will provide important insights into neuronal organization.

Functional consequences of heterotypic tiling

Avoidance of specific regions of the body wall may represent a mechanism utilized to coordinate positioning of dendritic/axonal arbors that comprise the sensorimotor circuitry. Consistent with this idea, the spatial pattern of motor neuron dendritic arbors in the Drosophila CNS correspond to the positions of the muscles they innervate (Landgraf et al, 2003). In the CNS, dendrites of motor neurons innervating internal muscles are restricted to the P compartment and dendrites of motor neurons innervating external muscles are present in the A compartment. Positional information is key to neuronal connectivity and function. Studies in the vertebrate spinal chord have shown the importance of motor neuron position, which influences its ability to synapse with the correct muscles (Jessell et al, 2011; Lek et al, 2010).

We propose that heterotypic tiling enables proper arrangement of dendritic fields in a cell-type specific manner ensuring that they receive and respond to stimuli that are unequally distributed across the body wall. It is possible that during locomotion (larval crawling), parts of the body wall innervated by proprioceptors contort differentially which leads to sensory feedback required for locomotion. In order to ensure that dendrites of neurons belonging to specific modalities can receive stimuli, which may be important during locomotion arbors of different types of neurons should be placed in appropriate parts of the body wall.

Assessing whether restriction of dendritic fields to distinct regions within the epidermis may have functional relevance will require new approaches for live imaging to correlate arbor deformation with peristalsis, as well as the ability to manipulate dendritic boundaries and assess the consequences for locomotion or tactile sensation. In conclusion, dendritic boundaries established by heterotypic tiling could be important for da neuron sensory function, a possibility that I test in chapter 4.

Cellular basis of homotypic tiling among class III neurons

In addition to identifying a new form of heterotypic tiling among sensory neurons, our results have relevance for understanding the mechanisms of homotypic (classical) tiling. Repulsive dendrite interactions between neighboring neurons are responsible for limiting dendritic fields and establishing tiling boundaries (Blackshaw et al, 1982), (Grueber et al, 2003b), (Sagasti et al, 2005). The role of homotypic repulsive interactions in limiting dendritic fields has been very well characterized in the class IV

da neurons (Grueber et al, 2003b). However, very little is known of the interactions that determine the dendritic borders class III neurons. Duplication of the different classes of neurons strongly suggested that dendritic fields of these classes of neuron are not entirely determined via repulsive interactions and different neuron types may establish their tiling boundaries by utilizing alternative mechanisms (Grueber et al, 2003b). Specifically, duplication of class III neurons resulted in partial overlap of major fields and only the higher-order dendrites showed evidence for repulsion (Grueber et al, 2003b). Our ablation studies provide evidence that support the overall conclusions of these prior studies. We find that although dendrites of the remaining (unablated) neurons showed a trend towards growth they do not significantly invade the territory that was once occupied by the ablated neuron. These results together with the duplication studies suggest that homotypic repulsive interactions are likely to refine dendritic tiling boundaries of class III neurons.

We note that the existence of additional cues (i.e. not solely contact mediated repulsion) that specify tiling dendritic fields is not without precedence. Evidence for additional mechanisms first came from ablation experiments performed in retinal ganglion cells of cats (Eysel et al, 1985). After widespread ganglion cell deletion, the remaining cells re-oriented and extended their dendrites towards the ablated region, but they failed to completely arborize the vacant space. Similarly, loss of most ganglion cells in Math5 or Brn3b mutants led to only modest increases in arbor territories among the remaining cells (Lin et al, 2004). Thus, whereas dendrite-dendrite interactions are clearly of primary importance for tiling in certain classes of

cells, other cell types are modestly affected and likely employ alternative cues. The identity of these cues remains unknown in any system and represents a critical future direction in the field. Our identification of an analogous type of tiling control in flies opens the question to further genetic analysis.

Figure 2.1 Dendrite arborisation (da) neurons have distinct dendritic fields and exhibit homotypic and heterotypic tiling interactions.

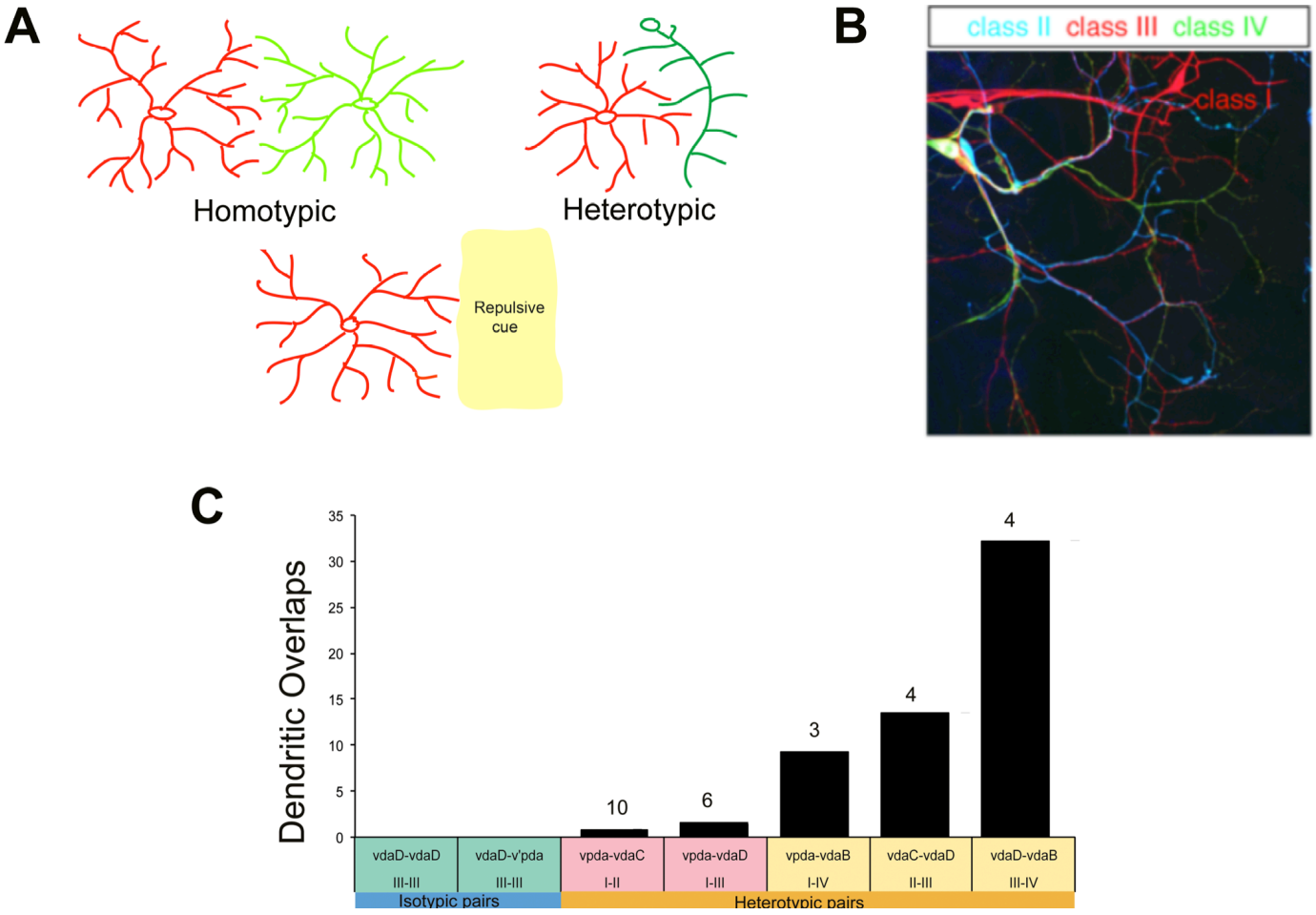


Figure 2.1 Dendrite arborisation (da) neurons have distinct dendritic fields and exhibit homotypic and heterotypic tiling interactions.

(A). Schematic of possible mechanisms for establishing dendritic fields.

(B) Different classes of da neurons are labeled using Flp- out system and ppk-eGFP. Class I (vpda) and class III (vdaD) labled in red, class II (vdaC) labeled in blue and class IV (vdaB) in green.

(C) The number of overlaps were quantified between pairs of neurons belonging to the same and different classes. Light blue bar indicates isoneuronal tiling, and orange bar indicates heterotypic tiling. The class assignments of the pairs are shown along the lower row, and pairs of neurons that show heterotypic tiling are highlighted.

Figure 2.2 Tiling boundaries are maintained throughout development

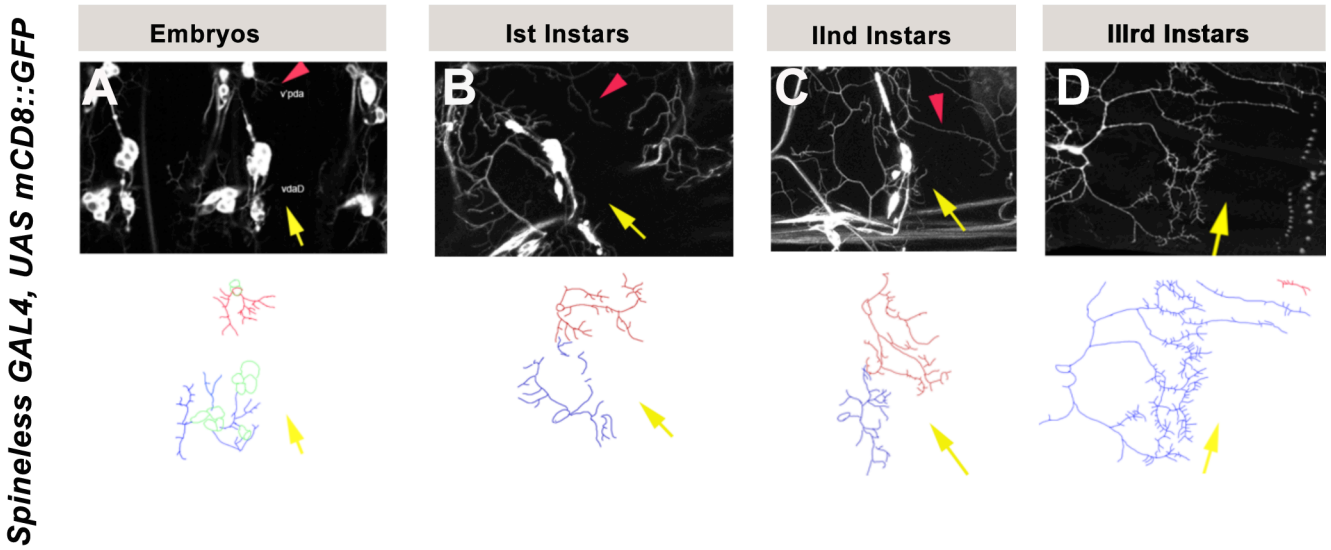


Figure 2.2 Tiling boundaries are maintained throughout development

(A) Ventral class III dendrites (vdaD) labeled with GFP using a class specific GAL4 (ss-GAL4) were visualized live in stage 17 embryos

(B) Class III dendrites (vdaD) labeled with GFP with ss-GAL4 were visualized live in I^{st} instar larvae

(C) Class III dendrites of vdaD labeled with GFP with ss-GAL4 were visualized live in II^{nd} instar larvae

(D) Class III dendrites of vdaD labeled with GFP using ss-GAL4 were visualized live in III^{rd} instar larvae

(A-D) Dendritic boundary of vdaD is established during the embryonic stages and remains unchanged during the larval stages.

Dorsal is up anterior is to left in figures A-D.

Genotypes: (A-D) spineless (*ss)- GAL4, UAS mCD8::GFP*

Figure 2.3 Role of homotypic tiling in class III neurons

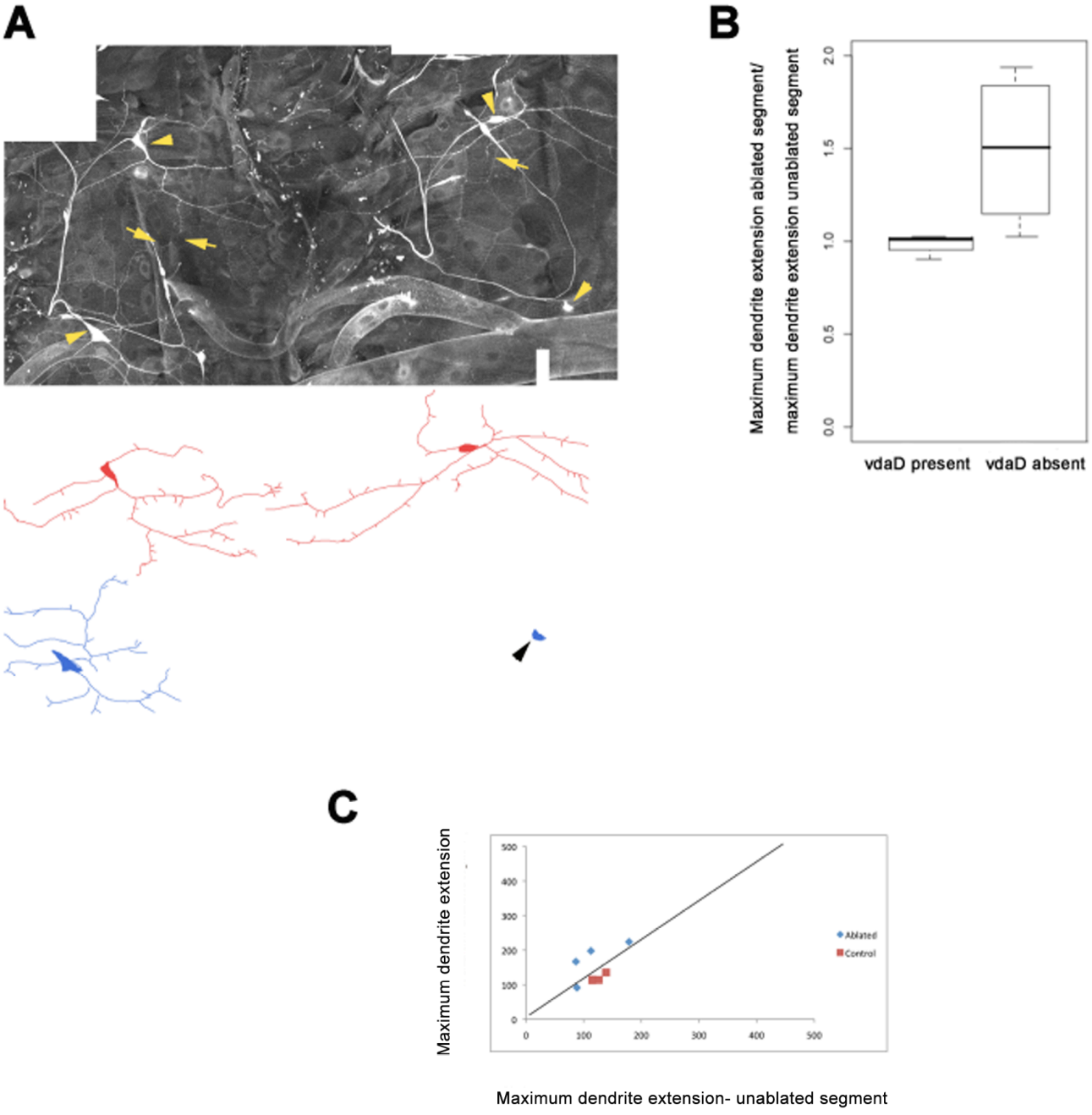


Figure 2.3 Role of homotypic tiling in class III neurons

(A) Laser ablation of class III neurons. No ablation was performed in leftmost segment and tiling restricts growth of red arbor. Laser ablation of class III neuron (right) leads to reorientation of red arbor into territory normally occupied by blue arbor.

(B) Quantification of the affects of laser ablation of class III neurons. A non-significant trend toward increased outgrowth was observed (ablated n= 6, unablated n=6).

(C) Ablation of vdaD shows slight growth v'pda dendrites towards the vacated space when compared to controls. V'pda dendrite growth in segments with ablated vdaD (blue), v'pda dendrite growth in segments where vdaD is present (pink) (ablated n= 4, unablate n=4)

Figure 2.4 Role of repulsive interactions in heterotypic tiling

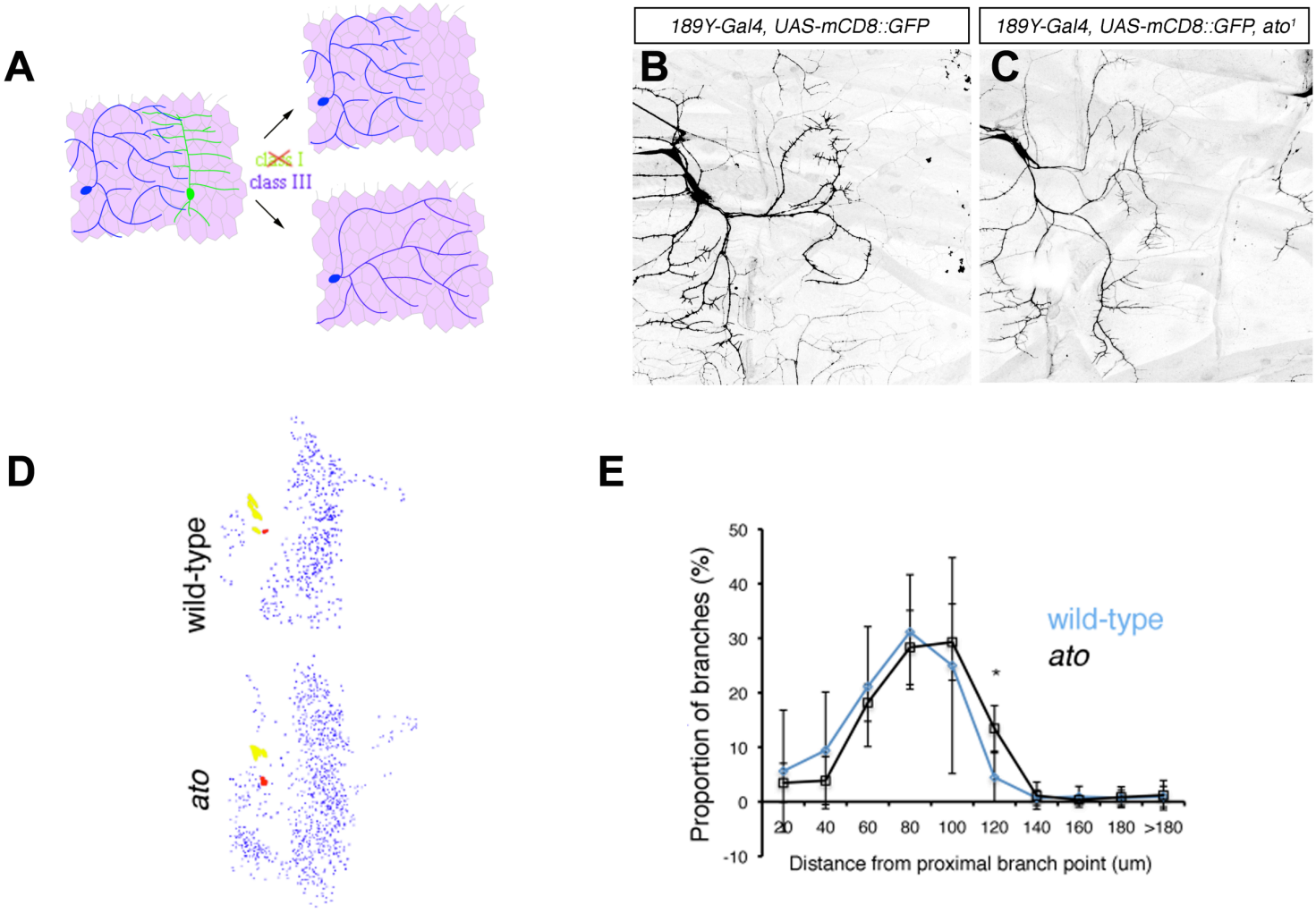


Figure 2.4 Role of repulsive interactions in heterotypic tiling

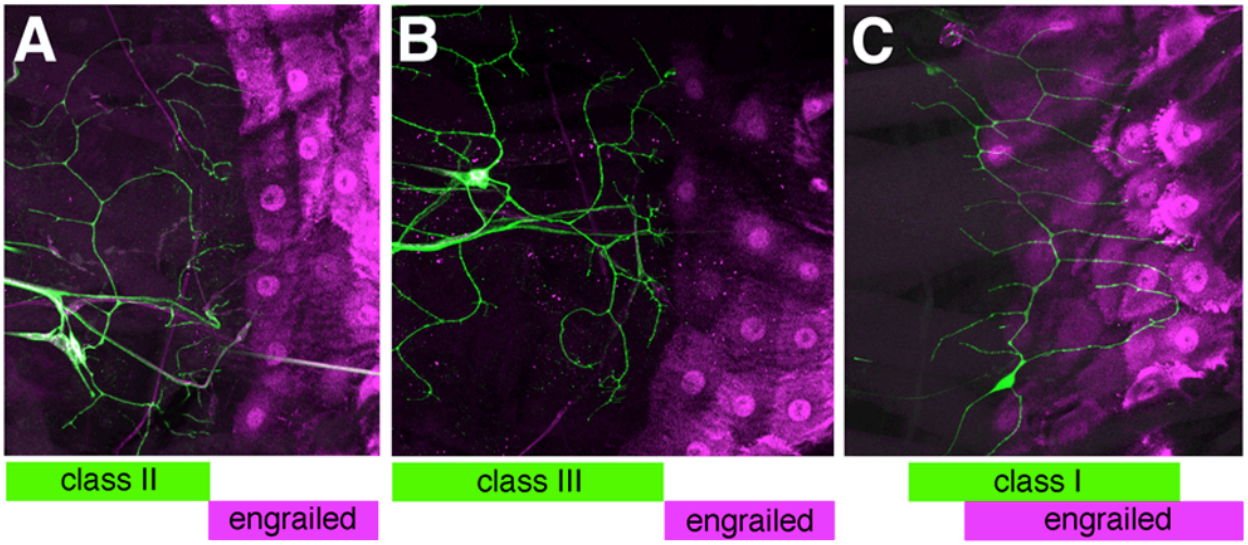
(A) Schematic representing alternative scenarios for class III dendrite of vdaD behaviors upon deletion of class I neurons. Class I and III neurons are colored differently: class I (vpda), green and class III (vdaD), blue.

(B,C) Mutations in *atonal (ato)* lead to genetic ablation of class I neurons (vpda). In the absence of the class I neuron, class III arbors of vdaD show little growth and do not invade the vacated area. (wildtype n= 7, *ato* n= 8)

(D) Extent of dendritic territories of class III neurons (vdaD) in the presence and absence of class I neuron vpda (wildtype n= 7, *ato* n= 8)

(E) Quantification of the effects of ablation of the class I neuron (vpda). The class III arbors of vadD do not arborize the territory formerly occupied by class I neurons (wildtype n= 7, *ato* n= 8)

Figure 2.5 Class specific patterns of organization of da neuron dendritic territories



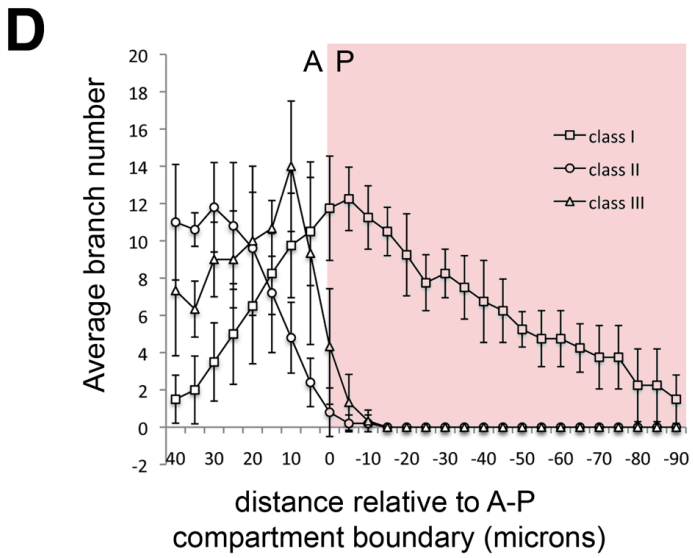


Figure 2.5 Class specific patterns of organization of da neuron dendritic territories

(A, B) Class II/III neurons avoids the posterior compartment labeled with *en-lacZ*

(C) Class I neuron primarily arborizes the posterior compartment labeled with *en-lacZ*

(D) Quantification of the average branch number of different classes of da neurons relative to the AP compartment boundary

Chapter 3

Dissection of molecular cues regulating dendritic boundary formation in *Drosophila* sensory neurons

Introduction

The establishment of proper dendritic territories determines the receptive field properties of neurons, however the factors that limit dendritic fields are not well-defined. One conserved mechanism for restriction of dendritic growth is tiling, in which dendrites from neighboring, usually functionally similar neurons, do not cross one another and thus evenly innervate all of their inputs. Tiling is widespread among Drosophila da neurons. Class IV neurons and class III neurons both innervate most of the epidermis in a non-redundant manner. In the case of class IV neurons, non-overlapping fields are set up by repulsive interactions between dendrites (Grueber et al, 2003b). In contrast, dendritic fields of class III neurons do not cover the entire epidermis, likewise the fields of class I and II neurons are smaller and non-contiguous. Because the boundaries of class I-III dendritic fields cannot be explained entirely by isotypic tiling, they likely require additional growth inhibiting or permissive cues from their environment.

So far we know very little about the molecular mechanisms that regulate dendritic boundaries. Studies in the fly visual system have identified molecular mechanisms of dendritic and axonal tiling. *Drosophila* R8 photoreceptor axons require heterophilic repulsive interactions mediated by the cell surface receptor *golden goal* (*gogo*) and an unkown ligand. As a result, adjacent *gogo* mutant R8 axons are mispositioned and axons overlap (Tomasi et al, 2008). Down syndrome cell adhesion molecule -2 (Dscam2) regulates the position of L1 lamina neurons (Millard & Zipursky, 2008), but unlike Gogo

it mediates positioning through homophilic interactions. Single *Dscam2* mutant axons fail to remain in their column and invade columnar territories of adjacent L1 neurons. The above studies in the fly eye have provided important initial understanding of the molecular mechanisms that establish tiling borders.

In contrast to these studies in the eye, membrane receptors that control tiling of sensory arbors are not known. My results in Chapter 2 suggest that class III neurons may utilize homotypic repulsion only to refine their dendritic territories. Here we test whether cues provided by overlying epidermal cells contribute to dendritic boundary formation, focusing on cues provided by epidermal compartments. The *Drosophila* epidermis is divided into alternating Anterior (A) –Posterior (P) compartments. The A compartment is defined by the expression of *patched (ptc)*, the receptor for the Hedgehog morphogen and the P compartment is defined by *engrailed (en)* and *hedgehog* (*hh*) expression. We have described in the previous chapter that class III (and class II) dendrites in the ventral epidermis arborize solely within the A compartment, whereas the class I neuron arborizes within the P compartment. In contrast to this compartmental restriction, class IV dendrites show no obvious compartmental preferences.

In this chapter I study the role of compartmental cues in dendritic boundary formation. My results suggest that Hh and its signaling components determine the routing of a class I primary dendrite along the AP compartment boundary, but apparently via a novel mechanism of signaling. Disrupting Hh pathway components causes the primary dendrite to shift its position away from the AP compartment boundary and into the P compartment, although the dendritic territory is largely preserved. In contrast to the dependence of class

I patterning on compartmental signals, class III dendrites showed no obvious responses to manipulation of compartmental signals, and may be determined by redundantly acting signaling pathways. Notably, prior studies identified a similar compartment organization for motor neuron dendrites in the CNS, however the molecular basis for this organization is not known. Similar molecular cues could therefore organize dendritic field boundaries in both the CNS and PNS (Landgraf et al, 2003).

Results

Class I territory specification by Hedgehog

To investigate the underlying molecular mechanisms regulating dendritic boundaries, we examined the possibility that dendrites receive signals from nearby epidermal cells. Previous studies have shown that da neuron dendrites contact the basal membrane of epidermal cells, suggesting potential signaling between the epidermis and sensory neuron dendrites (Kim et al, 2012a), (Han et al, 2012). We showed previously that dendrites obey segmental boundaries (Chapter 2). I first focused on understanding the signals that produce the stereotyped field of the ventral class I neuron, vpda. During embryonic development, vpda grows a primary dendrite in a dorsal direction along the boundary of the A-P compartment. Short secondary dendrites extend from this main dendrite in an anterior direction within the field of Ptc-expressing epidermis, and longer secondary dendrites extend posteriorly within the P-compartment.

Given the correlation between molecular and dendritic boundaries we hypothesized that the growth of class I dendrites could be biased by Hh signaling. Preliminary

results indicated that when Hh was misexpressed in Wg-positive A compartment cells (Fig 3.1 A, B), dendrite polarity of class I neurons was altered (J. Lee, Y. Lei, and W. Grueber, unpublished). To quantify the effect of Hh on dendrite polarity I measured the ratio between the class I dendrites anterior to and posterior to the primary dendrite (for details see Materials and Methods). We termed this ratio the asymmetry index (a.i.), where a positive a.i. represents anterior polarization of class I secondary dendrites, a negative value represents posterior polarization of secondary dendrites, and a value of 0 indicates a symmetrical field of secondary dendrites. My analysis showed that there was a significant switch of polarity in class I secondary dendrites in animals over-expressing Hh ($n = 12$, p-value = 0.003, Fig 3.1 C, D). Thus, when Hh is expressed ectopically in A compartment cells, class I neurons show relatively longer secondary dendrites extending toward the A compartment. I confirmed that this effect was not likely due to a decreased overall dendritic growth by measuring total dendrite length of these neurons. I found that the total dendritic length remained unchanged (p-value = 0.83).

In principle two alternative scenarios could account for the observed change in dendrite polarity, with each leading to a distinct interpretation of the role of Hh in dendritic field positioning. First, a shift in polarity could result from an increased invasion of dendrites into the A compartment, which would indicate a positive role for Hh in dendrite growth. Second, a similar quantitative result could arise from posterior re-positioning of the primary dendrite in relation to the AP compartment boundary, in which case Hh could be seen as having a “repulsive” effect on the primary dendrite

and no effect on secondary dendrite growth (Fig. 3.1 E). To discriminate between these scenarios I mis-expressed Hh in the Wingless compartment in animals carrying an *en-lacZ* transgene to label the P compartment. The polarity vpda dendrites was quantified and, consistent with the results above, arbors exhibited a positive a.i. I next assessed the position of the primary dendrite relative to the AP compartment boundary. To quantify the position of the primary dendrites with respect to the AP compartment boundary I devised a quantitative method that sums the length of secondary dendrites between the AP compartment boundary and vpda primary dendrite. I termed the final sum the dendrite position index. This analysis revealed that the primary dendrite of vpda shifted posteriorly away from the AP compartment boundary and into the P compartment (Fig. 3.1 F, n = 7, p-value = 0.001). I conclude based on this analysis that misexpression of Hh in the A compartment causes a shift of the primary dendrite into the P compartment from its normal position along the A-P compartment boundary, and has little or no effect on the position of the "footprint" of the vpda dendritic field.

Patched is required by vpda to respond to Hedgehog

The experiments above indicate that Hh over expression can regulate dendrite patterning. To examine whether the Hh pathway affects vpda patterning, I examined the role of downstream components of the Hh pathway through which Hh regulates primary dendrite positioning. Patched (Ptc) is a twelve-pass transmembrane receptor that normally acts to inhibit the activity of Smoothened (Smo). Binding of Hh to Ptc

relieves Smo from its repressed state, which leads to activation of the transcription factor Cubitus interruptus (Ci). Thus in the absence of Ptc the Hh pathway is constitutively active. Given the logic of this pathway I examined whether Hh regulates vpda primary dendrite positioning by transducing its signal through Ptc. In order to test this idea, I examined the position of the primary dendrite of vpda in *ptc* mutant MARCM clones. If the Hh signal is transduced through the Ptc receptor I expected to see a posterior shift in the position of the primary dendrite similar to that observed upon ectopic expression of *hh* in the Wg compartment. I generated and examined ptc^{IIW} and ptc^{S2} MARCM clones (Fig. 3.3 A-C). In ptc^{IIW} mutants neurons, no Ptc protein is produced and in ptc^{S2} neurons, a mutant Ptc protein is encoded, which localizes on the cell surface but behaves like a *ptc* mutant (i.e it is unable to inhibit Smo activity and leads to ectopic activation of the Hh pathway). I quantified the a.i and primary dendrite position of ptc^{IIW} and ptc^{S2} MARCM clones. ptc^{IIW} vpda clones showed a significant shift in their dendrite polarity and had a positive a.i. (Fig.3.3 D, n =7, p-value = 0.001). Likewise, similar to the effect seen in animals over-expressing *hh*, the primary dendrite of ptc^{IIW} clones shifted away from the A-P compartment boundary and into the P-compartment (Fig. 3.3F, n = 6, p-value = 0.001). In contrast to these results, ptc^{S2} MARCM clones did not exhibit a shift in a.i or in primary dendrite position when compared to WT clones (Fig. 3.3 E, G, n= 7 p-value = n.s. (ptc^{IIW} compared to ptc^{S2}) = n.s.). Thus, Ptc is important for dendrite positioning, but cell surface localization of a Ptc that cannot inhibit Smo appears sufficient to rescue the full function of the protein.

Smoothened is required for positioning of the vpda primary dendrite along the A-P compartment boundary

Hh over-expression and *ptc* mutant studies suggest that the Hh pathway regulates vpda primary dendrite position along the AP compartment boundary. Hh signaling can follow either canonical or non-canonical routes. Canonical signaling involves Hh signal transduction through Smo, whereas non-canonical signaling occurs via Ptc independent of Smo. To investigate whether the canonical downstream components of Hh pathway regulates vpda primary dendrite position, we examined the requirement of *smo* by generating smo^{3} mutant MARCM clones (Fig. 3.2A,B). If the Hh pathways acts through Smo to regulate primary dendrite position we would expect an opposite phenotype to that of ptc^{IIW} clones, such that the secondary dendrites may polarize towards the P compartment and the primary dendrite of vpda may shift anterior to the AP compartment boundary. I quantified a.i. and primary dendrite position and found that, similar to the ptc^{IIW} neurons, the a.i. index of smo^{3} neurons revealed a bias towards the A compartment (Fig. 3.2 C, n=5, p-value 0.001). I could not generate clones with the P compartment labeled, so in order to assess the position of the vpda primary dendrite I measured the distance between the ventral ch organ (which resides in the A compartment) and the first point of contact that the primary dendrite makes with the epidermis (Fig. 3.2D). I verified that this is a valid method of assessing primary dendrite positioning by examining the distance between the AP compartment boundary and the vch organ in ptc^{IIW} and wildtype clones where the P compartment was labeled with en-lacZ . I found that the distance between ventral Ch organs and

primary dendrites of vpda increased compared to wildtype clones, which suggested like in the case of ptc^{IIW} neurons there was a posterior shift in the position of the primary dendrite (Fig. 3.2.D). This result was unexpected as smo^{3} mutant neurons lack the ability to transduce and respond to the Hh signal. One explanation might be that smo^{3} clones, like in the case of ptc^{IIW}vpda clones, are unable to transcriptionally activate *ptc*. As a result the level of Ptc on smo^{3} vpda neurons is reduced.

These results reveal that loss of either Smo or Ptc causes similar changes in primary dendrite positioning of vpda.

Cubitus interruptus regulates vpda primary dendrite positioning

Cubitus interruptus (Ci) is a zinc finger transcription factor that is activated as a result of Hh signaling. It is present in an inactive form in cells and upon receiving Hh signal it is activated and translocates to the nucleus to transcriptionally activate *ptc* and numerous other target genes. My data so far suggests a role for Hh signaling in determining the position of vpda primary dendrite along the AP compartment boundary, which led me to ask whether the Hh signal is transduced through Ci. To test this idea I over-expressed *ci*- activated (ci^{Act}) and repressor (ci^{R}) forms in da neurons. Neurons that express the ci^{Act} form behave as if they are always receiving Hh signal. Conversely, ci^{R} inhibits Hh signaling and prevents target genes from being transcribed. If the Hh signal is transduced through Ci, I would expect that misexpression of ci^{R} in vpda would block Hh signaling and the phenotype should be similar to *smo* clones. In

the case of ci^{Act} misexpression, the Hh should be constitutively active, which should lead to the transcription of genes such as *ptc*. Here, we could see that the cells maintain their normal polarity and primary dendrite position as there is a high level of Ptc on the surface of the neuron.

I used a pan-neuronal GAL4 driver, *109(2)80 GAL4::UAS mCD8 GFP* to over-express the two different forms of *ci* (Fig 3.4 A-C). Similar to vpda clones that lack Smo and Ptc function, neurons expressing ciR showed a positive a.i. (Fig. 3.4D, n= 7, p-value = 0.01) and a posterior shift in the primary dendrite position (Fig. 3.4E). By contrast, over-expression of the activated form did not change the polarity of vpda dendrites, nor alter the position of the primary dendrite (Fig. 3.4D,E, n= 6 , p-value = n.s.). In conclusion, my data suggest that Hh, acts through Ptc, Smo and Ci to specify the positioning of the class I primary dendrites with respect to the AP compartment boundary. Notably, the precise positioning of secondary dendrites along the body wall was unaffected by any of my manipulations of the Hh pathway, suggesting separable control of different orders of dendritic branches.

Misexpression of Knot over-rides the restriction of class III dendrites to the A compartment

Class III and class II dendritic territories in the ventral cluster occupy a region that is complementary to, and non-overlapping with, class I dendritic territories. However ablation studies indicated that interactions between class II/III and class I dendrites are

alone unlikely to specify these boundaries. To gain insight into mechanisms that specify class III boundaries, I first asked whether the boundary of ventral class III neuron vdaD is determined by intrinsic mechanisms. The transcription factor Knot is specifically expressed in class IV neurons and is necessary and sufficient for their characteristic branching patterns. Notably, in contrast to class III neurons, Knot-positive class IV neurons show no compartment preferences, thus I asked whether misexpression of *kn* in class III neurons could alter their compartment preference and lead to the invasion of dendrites into the P compartment. I misexpressed *knot* using a pan-neuronal driver *109(2) 80 GAL4, UAS mCD8:: GFP* while simultaneously labeling the P compartment with *en-lacZ* (Fig 3.8 A,B). I measured the total length of vdaD dendrites present in the P compartment and found that dendritic growth into the P compartment increased significantly in vdaD neurons expressing the *UAS-knot* transgene compared to control neurons not expressing *UAS-knot* (Fig. 3.8 C, n= 5, p-value = 0.001). This result suggests that class-specific compartment preferences are determined by cell-intrinsic programs. Knot could conceivably control the expression of specific receptors that allow the cell to respond to extrinsic cues that control territory preferences.

It is conceivable that Knot could simply promote arbor growth, and that overgrowth of arbors is sufficient to allow invasion of class III arbors into the P compartment. To test this idea, I asked whether simple over-expression of growth factors in vdaD was sufficient to cause dendrites to grow into the P-compartment. I tested three known growth factors, insulin like receptor (*in(R)*), *akt*, Ras homolog brain enriched ortholog

(*rheb*) (Fig. 3.8b D-G). Over-expression of these genes did not result in extra elaboration of class III dendrites with the possible exception of *rheb*. However, cells overexpressing *rheb* still did not project dendrites into the P compartment. This result suggested that intrinsic dendrite growth capacity is not the limiting factor restricting vdaD dendrites to the P compartment, and suggest instead that responses to compartmental cues underlie dendritic field boundaries.

Examination of axon guidance receptor involvement in dendritic compartment preferences

I next focused on identifying potential cues from the substratum that set the dendritic boundaries of vdaD neurons. Initial efforts in the lab focused on identifying cues that, when misexpressed in class IV neurons, caused dendrites to terminate within the A compartment. Preliminary results indicated that overexpression of the Ig superfamily member Robo3, but not Robo1 or Robo2 could cause class IV dendrites to terminate entirely within the A compartment (Fig. 3.5 A-C) (Lee, J., unpublished). To examine whether this was a specific effect of Robo3 I overexpressed additional receptors using the same assay. I found that *unc-5*, *sema 1a*, *plexB* and *ephrin* were not sufficient to restrict class IV dendrites to the A compartment (Fig. 3.5 D-F).

Next, I asked whether *robo3* was required by vdaD to restrict dendrites to the A compartment. If this is the case then removal of *robo3* should lead to an over-growth of the dendrites to the P compartment. To test this possibility, I generated *robo3*

MARCM clones of vdaD neurons (Fig. 3.6 A-B). I quantified the possible change in their compartment preference by generating clones in a *hh-lacZ* background. *hh-lacZ* like *en-lacZ* is a reliable marker for the P compartment. I quantified the change by measuring the total length of dendrites in the P compartment in both the control and mutant neurons and found that there was no significant change in the average dendritic lengths (p-value = 0.62). This result suggests that *robo3* alone is not required for restricting vdaD dendrites to the A compartment. I confirmed these results by examining embryos and third instar larvae mutant for *robo3* (Fig. 3.6 C-E; n= 8, p= 0.7). I also examined neurons mutant for different combinations of Robo receptors. Likewise, a triple mutant combination of (*robo 1,2,3*) did not cause dendrites to grow into the P compartment, nor did misexpression of Commissureless, a protein that prevents localization of Robo receptors to the cell surface (Fig. 3.7 A, B). Together, these results suggest that Robo3 alone is not required for restricting the dendritic boundaries of vdaD to the A compartment, but may be redundant with other Robo-independent signals.

I reasoned that one possible Robo-independent redundant signal could be a repulsive signal from vpda dendrites. In this scenario, dendrite-dendrite interactions and dendrite-substrate signaling could together regulate vdaD boundary formation. To address this question I investigated *robo3;ato* mutant animals. In robo3;ato mutants vpda is never specified and cells will also not be able to respond to signals via Robo3, so if these mechanisms act in concert this manipulation should result in over-growth of vdaD dendrites. To the contrary, vdaD dendrites in robo3,ato mutants did not show

significant over-growth. These results suggest that Robo3 and heterotypic tiling interactions between vpda-vdaD do not play a redundant role in regulating vdaD dendritic boundaries.

Class III dendrites do not respond to ectopic sources of Engrailed

Prior studies from the lab demonstrated that dendrites of vdaD avoid crossing over P compartment epidermal cells, which are defined by the expression of Engrailed (En). I next tested whether expression status of En was sufficient to determine whether vdaD dendrites were capable of innervating epidermal cells. I expressed *en* ectopically in a variable subset A compartment cells using a flip-out approach. In this method hsFLP induction leads to excision of a ubiquitously expressed CD2 stop cassette and permits expression of *GAL4*. This approach often led to a small number of A compartment cells expressing both *UAS-en* and *UAS mCD8::GFP* (Fig. 3.8 A-B). I counted the total number dendrites that grew across GFP-positive epidermal cells and divided by the area of the cell, which gave me a measure of the number of crossings/area. I found that the total number of dendrites growing on *en* expressing A compartment cells compared to wildtype cells in the A compartment did not change significantly (Fig. 3.8C (n=5, p-value= 0.99).

Classic boundary cues and guidance molecules do not affect dendrite positioning of class III dendrites

Lastly, I examined numerous other candidate molecules for their role in restrictiong vdaD dendrites to the A compartment using loss of function approaches in stage 12-15 mutant embryos. I simultaneously labeled class III dendrites using class III specific GAL4 driver, spineless (*ss)- GAL4, UAS mCD8::GFP* (Fig. 3.9 A- C). If a candidate molecule is required for restricting vdaD dendrites to the A compartment then removal of the molecule should result in the invasion vdaD dendrites into the P compartment. In this screen, I examined a total of ten compartment and signaling cues (Fig. 3.9D) and found that none of these molecules lead to the invasion of vdaD dendrites into the P compartment. In all mutant embryos both the overall class III dendritic pattern and boundaries remained unperturbed (Fig. 3.9 A-C). My findings corresponds to the studies performed to investigate the molecular mechanisms patterning motor neuron dendritic territories in *Drosophila* CNS, where like in the PNS classic boundary cues do not influence dendritic borders (Landgraf et al, 2003). These data together suggest that classical boundary cues and guidance molecules are not responsible for restricting vdaD dendrites to the A compartment. Despite some initial evidence for a specific role for Robo3 in setting dendritic boundaries, my loss of function analysis suggests that establishment of dendritic domains of class III neurons is very robust and is likely to be regulated by multiple redundantly acting signaling pathways.

Discussion

Defining mechanisms that establish dendritic territories will provide insights into appropriate sampling of sensory information and neural circuit assembly. So far we know very little about how dendritic fields of neurons are determined by potential signaling cues from the substrate. To address this question I investigated whether compartment specific organization of da neurons is regulated by classical morphogen and guidance cues that originate in specific compartments and if they provide positional information that may be important for establishing dendritic territories. Positional information is key to neuronal connectivity and studies in the vertebrate spinal chord have demonstrated the importance of motor neuron position in forming synapses with appropriate muscles (Lek et al, 2010), (Jessell et al, 2011). Compartment specific cues have been shown to play a role in positioning neurons in the adult *Drosophila* abdomen (Fabre et al, 2010). In addition, midline guidance cues such as Robo and Netrin have been shown to be involved in positioning of motor neuron dendrites in the leg neuropil (Brierley et al, 2009). Altogether, studies of sensory axon positioning in *Drosophila* CNS demonstrate the complex interactions that occur between different guidance systems to precisely position axons (Zlatic et al, 2003),(Wu et al, 2011),(Zlatic et al, 2009). My findings in this chapter have suggested that morphogens and guidance cues might act in a redundant manner to regulate dendrite positioning and territories.

A novel role for patched in determining dendrite patterning

Our initial *hh* over-expression experiments led to a strong alteration in the polarity of class I dendrites (vpda). Closer examination revealed that the shift primarily reflected a change in the position of the primary dendrite of vpda and that the overall dendritic territory position remained unchanged. As primary dendrites shifted into the posterior compartment the anterior secondary dendrites of vpda grew longer, compensating for the shift. Likewise the posterior dendrites decreased in length thereby maintaining the vpda dendritic boundary. I observed the same phenomenon in ptc^{IIW} and smo^{3} null mutants. This result was surprising as Ptc antagonizes Smo function, however I argue that in the absence of *smo* additional *ptc* is not transcribed and, as expected, in *ptc* mutants no Ptc is produced. Thus in both cases Ptc was not present at wildtype levels. However, in ptc^{S2} clones, where Ptc protein can localize to the cell surface but is unable to inhibit Smo activity, I saw no change in the primary dendrite position. These results together suggested that Ptc levels on the cell surface may regulate primary dendrite positioning.

One interpretation of these results is that dendrite positioning requires a novel function for Ptc that is independent of the Hh signal. In order to test whether the primary dendrite position of vpda is determined by Ptc receptor function that is independent of the Hh signal, one would need to show rescue of *ptc* clones with a *ptcloop2* construct. The *ptcloop2* construct lacks the ability to bind to Hh and if *ptcloop2* over-expression

in a ptc^{IIW} clone can restore primary dendrite position along the AP compartment boundary it would suggest that Hh signal is not required, and that Ptc may function independently of Hh to regulate primary dendrite positioning of vpda.

Numerous studies in *Drosophila* have suggested that different levels of Ptc on the surface of cells may mediate varied responses to Hh signaling (Johnson & Tabin, 1995), (Chen & Struhl, 1996), (Ma et al, 2002), (Chou et al, 2010). In particular, axon targeting studies in the Drosophila olfactory system have provided evidence that different levels of Ptc receptor in populations of olfactory receptor neuron (ORN) axons impart differential responsiveness to Hh signaling to mediate targeting to the brain. However, no study so far has described a Hh independent role for the Ptc receptor and so my results suggest a possible novel requirement for Ptc independent of Hh.

In addition, my data also suggest that there can be distinct mechanisms for patterning of primary and secondary dendrites of neurons. These differences could arise because the timing of primary and secondary dendrite outgrowth or branching coincides with temporally distinct signals and morphogen gradients.

Class III dendrites do not utilize classic boundary cues and signaling molecules to organize their territories

One key question in developmental biology is how developing cells receive positional information. Compartment cues and guidance molecules provide essential cues for organizing different cells and tissue types during development (Torroja et al, 2005). Initial over-expression experiments suggested that Robo3 may play a role in restricting vdaD dendrites to the A compartment. However, further examination of *robo3* mutant vdaD neurons suggested that either *robo3* was not required for this restriction or may act redundantly with other signaling molecules. I did not identify other candidate cues through loss of function or gain of function approaches, suggesting that either novel molecules are required for establishing class III dendritic boundaries, or that a suite of molecular cues act in a redundant manner.

One major question for further study is whether the signal that restricts class III dendrites to the A compartment is a repulsive cue from the P compartment or an attractive cue from the A compartment. At this point, my data cannot discriminate between these possibilities. Some preliminary evidence points to the latter scenario, and this is worthy of following up. Specifically, prior studies suggested that in the absence of self-repulsive signaling, class III dendrites tend to clump together in regions of attractive guidance cue expression (Matthews & Grueber, 2011). Preliminarily, I observed that in *Dscam* mutant clones of vdaD neurons anterior and posterior dendrites showed a clumping phenotype that was focused on a narrow band of cells in the A compartment, adjacent to the A-P compartment boundary in the Ptc-

expressing domain. Thus it is possible that an attractive cue may exist in the Ptc compartment that restricts dendrites to the A compartment and that a combination of repulsive and attractive cues in the substrate regulate dendritic boundaries of neurons

One strategy to identify molecule(s) that regulate repulsive/ attractive interactions with class II/III dendrites would be to profile P compartment cells and Ptc cells. Molecular profiling of P and Ptc cells may help uncover novel ligands that restrict class II/III dendrites to the A compartment.

The role of intrinsic mechanisms in dendrite patterning

Studies conducted in mammalian retinal neurons have demonstrated the intrinsic capacity of neurons to establish stereotypic dendritic territories in absence of input from the environment or neighboring neurons (Montague & Friedlander, 1991). These and other earlier studies performed on neurons in isolation provided preliminary evidence that in a subset of neurons dendritic territory can be genetically determined. Masland and colleagues confirmed this finding in vivo in two classes of ganglion cells (Lin et al, 2004). They studied *Brn3b*$^{-/-}$ and *Math5*$^{-/-}$ mice in which nearly 80-85% of tiling ganglion cells are absent and they found that the remaining cells failed to expand into the territories that would have been normally occupied by their neighbors. These studies raise the question whether the identity of a cell pre-determines its dendritic territory? Each class of da neurons has a unique transcription profile (Corty et al, 2009; Grueber et al, 2003a; Li et al, 2004; Sugimura et al, 2004). Class II-IV express different levels of Cut (Grueber et al, 2003b). Class I specifically expresses the

transcription factor Abrupt (Corty et al, 2009), (Jan & Jan, 2010) and only class IV neuron express Kn (Crozatier & Vincent, 2008), (Hattori et al), (Jinushi-Nakao et al, 2007). My data showed, dendrites of class IV neurons have no compartment preference and arborize the entire segment. Furthermore, when I misexpressed *kn* in class III neurons their dendrites invaded and arborized the P compartment. These results suggested that the molecular identity of a cell may play a role in compartmental organization of different classes of neurons. Cell specific transcription factors may regulate the expression of appropriate receptors that allow the neuron to respond to cues in the environment. My studies so far suggest that the identity of a neuron defined by the transcription factors it expresses and the substrate that it grows on are important determinants of dendritic boundary. Similar to the sensory dendrites, the territories of motor neuron dendritic arbors in the CNS also obey A and P compartment boundaries. In addition we examined the compartment specificity of da neuron axons in the CNS. Our preliminary studies suggest that in a subset of the da neuron axons respect compartment boundaries (Singhania, A., Grueber, W., unpublished results). The molecular basis of motor dendrite positioning is not known, but this coincidence raises the possibility that similar patterning cues may operate in the CNS and PNS, possibly functioning to bring central and peripheral arbors into spatial register with one another. It's conceivable that compartmental organization might operate at several levels to organize sensorimotor circuitry.

Acknowledgements

We would like to thank Ya-ting Lei and Jennifer Lee for performing the initial experiments on the effect of Hh overexpression on class I arbors. We are very grateful to Gary Struhl for his expertise, insight and fly stocks for studies of class I neurons. We thank Larry Zipursky for the *robo 1*,*2* and *3* stocks.

Figure 3.1 Hedgehog redistribution resets class I polarity by altering the position of the primary dendrite.

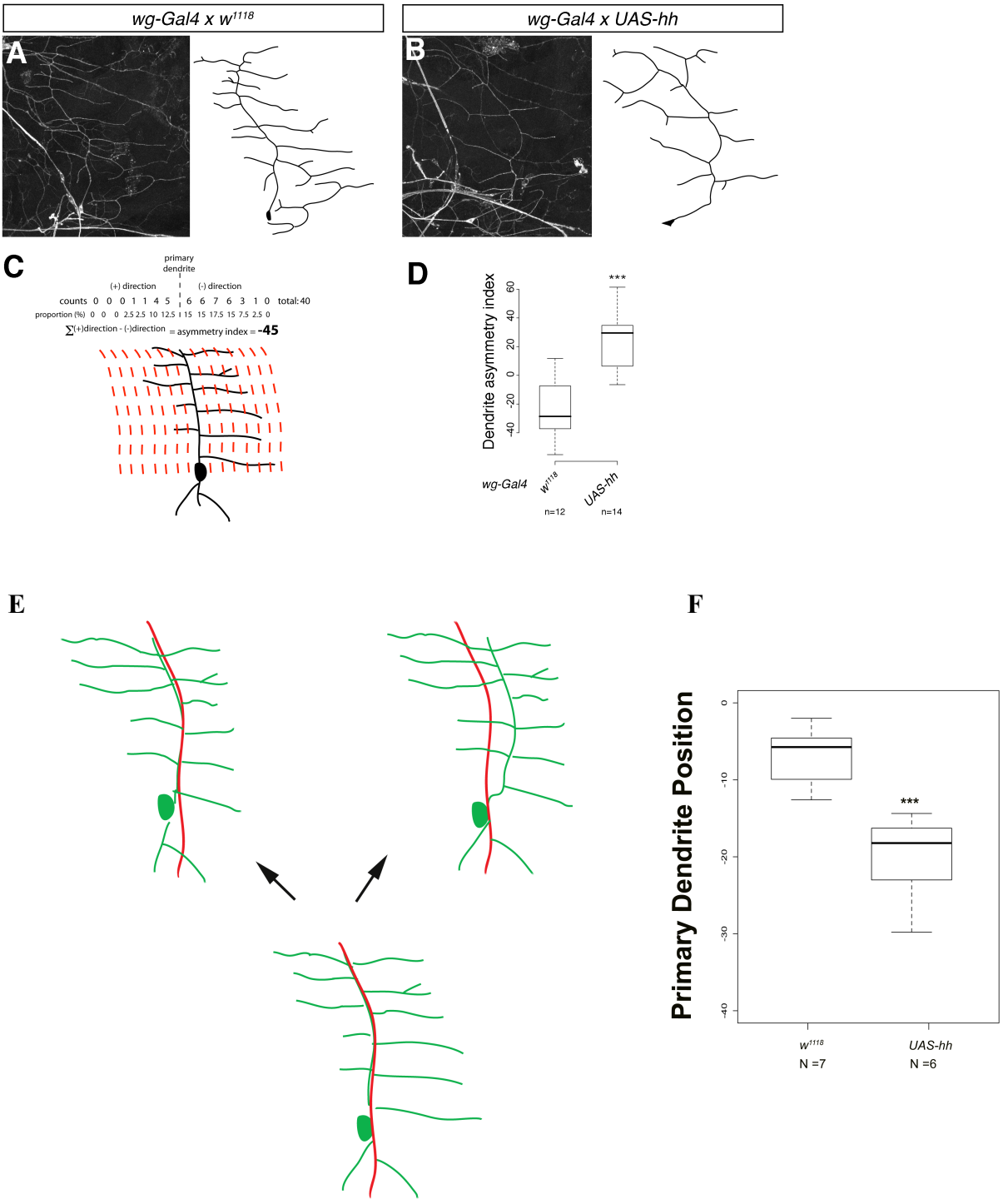


Figure 3.1 Hedgehog redistribution resets class I polarity by altering the position of the primary dendrite.

(A) HRP immunorecativity labels neuronal dendrites in *wg-GAL4* larvae

(B) HRP immunorecativity labels neuronal dendrites in larvae over-expressing *hh* in the Wg compartment

(C) Schematic of the a.i. analysis used to determine dendrite polarity

(D) Quantification of dendrite polarity in WT and *wg-GAL4* x *UAS hh* larvae

(E) Two alternative scenarios affecting dendrite polarity

(F) Quantification of the primary dendrite polarity with respect to the AP compartment boundary

Figure 3.2 Smoothened is required by class I neurons

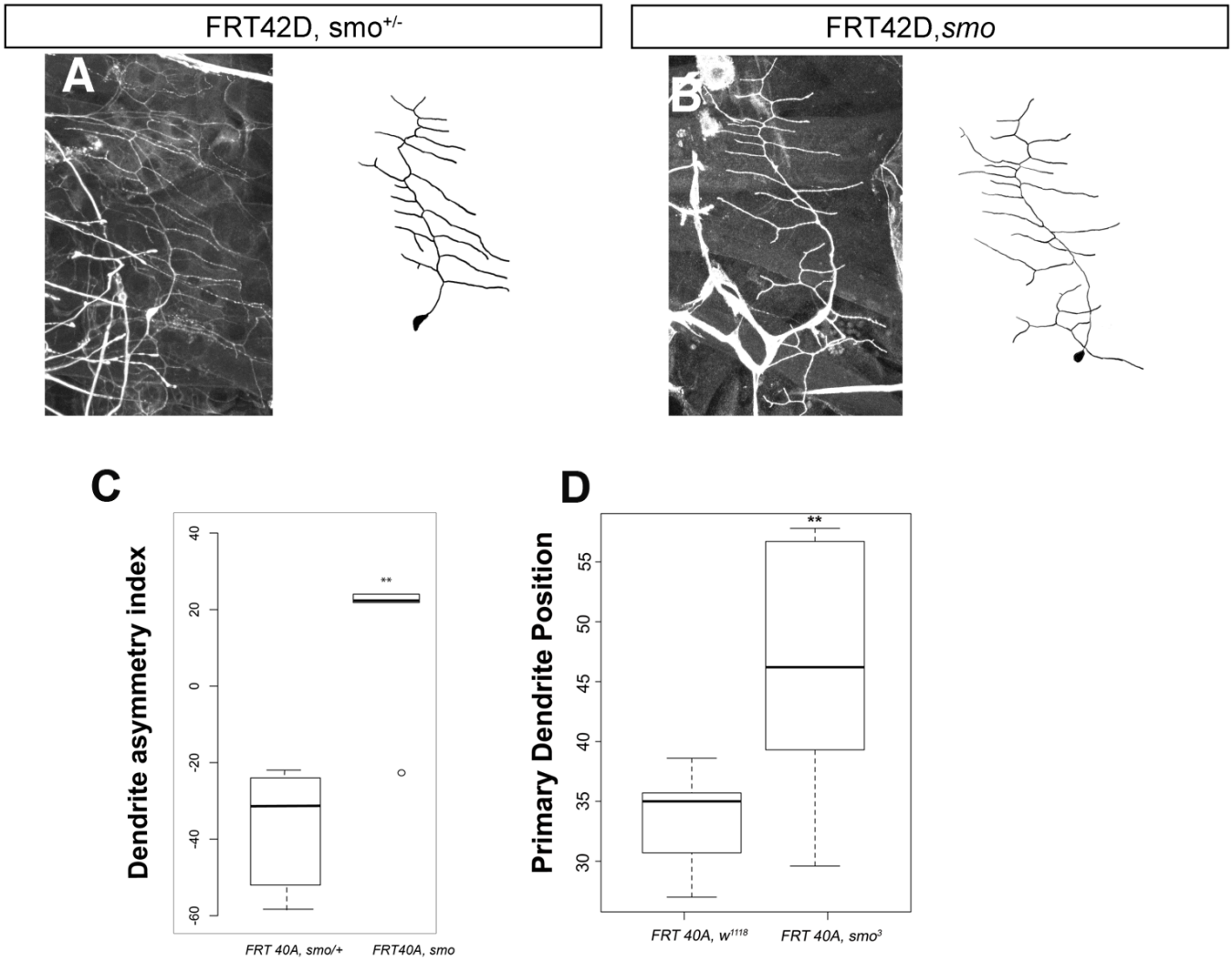


Figure 3.2 Smoothened is required by class I neuron primary dendrite position

(A) GFP immunorecativity labels neuronal dendrites in *smo* $^{+/-}$ larvae

(B) GFP immunorecativity labels neuronal dendrites in *smo* $^{-/-}$ larvae

(C) Quantification of dendrite polarity in *smo* $^{+/-}$ and *smo* $^{-/-}$ larvae

(D) Quantification of the primary dendrite polarity in *smo* $^{+/-}$ and *smo* $^{-/-}$ with respect to the AP compartment boundary

Figure 3.3 Patched is required for the positioning of primary dendrite

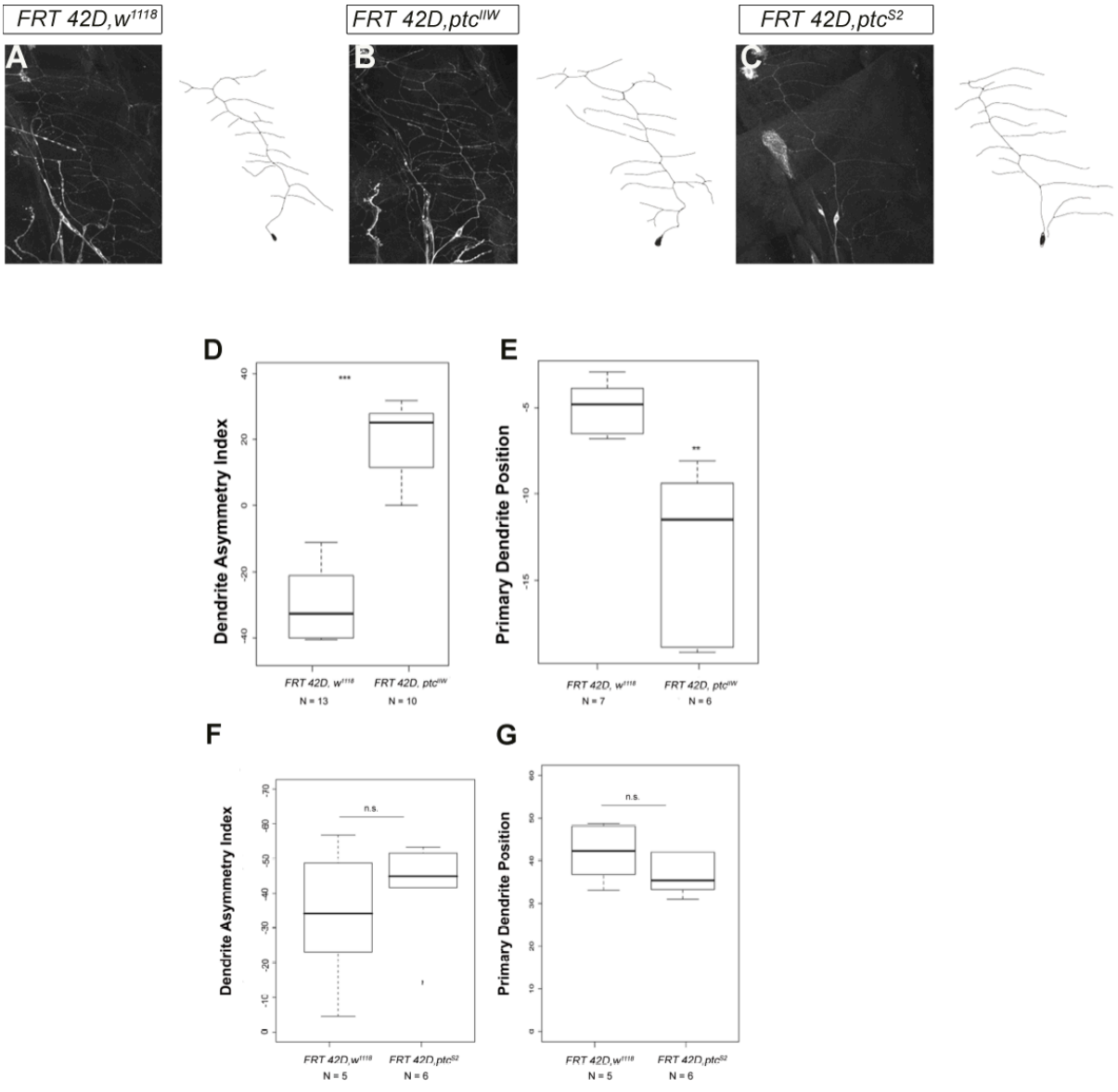


Figure 3.3 Patched is required for the positioning of primary dendrite

(A) GFP immunoreactivity labels neuronal dendrites in wild type larvae

(B) GFP immunoreactivity labels neuronal dendrites in *ptc* ^{IIW} larvae

(C) GFP immunoreactivity labels neuronal dendrites in *ptc* ^{S2} larvae

(D) Quantification of dendrite polarity in wild type and *ptc* ^{IIW} larvae

(E) Quantification of the primary dendrite polarity in wild type and *ptc* ^{IIW} with respect to the AP compartment boundary

(F) Quantification of dendrite polarity in wild type and *ptc* ^{S2} larvae

(G) Quantification of dendrite polarity in wild type and *ptc* ^{S2} larvae

Figure 3.4 Hedgehog determines primary dendrite position by regulating Ci activation

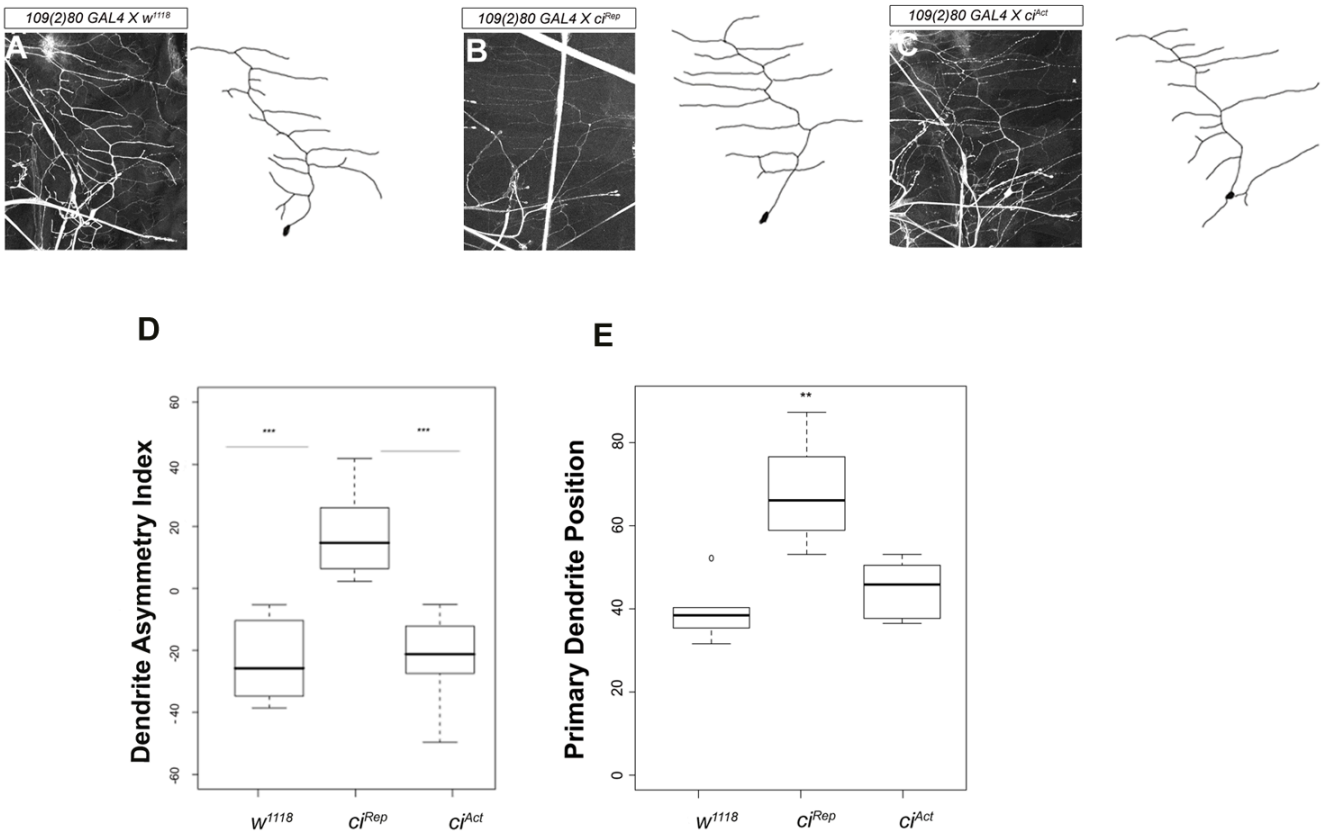


Figure 3.4 Hedgehog determines primary dendrite position by regulating Ci activation

(A) GFP immunorecativity labels neuronal dendrites in *109(2)80 GAL4,UAS CD8:: GFP* larvae

(B) GFP immunorecativity labels neuronal dendrites over-expressing ci^{R} pan-neuronally

(C) GFP immunorecativity labels neuronal dendrites over-expressing ci^{Act} pan-neuronally

(D) Quantification of dendrite polarity in wild type, *109(2)80 GAL4, UAS mCD8 :: GFP;ci* ^{R} and *109(2)80 GAL4, UAS mCD8 :: GFP ci* ^{Act} larvae

(E) Quantification of primary dendrite position in wild type, *109(2)80 GAL4, UAS mCD8 :: GFP;ci* ^{R} and *109(2)80 GAL4, UAS mCD8 :: GFP ci* ^{Act} larvae

Figure 3.5 Robo3 receptor induces anterior compartment restriction

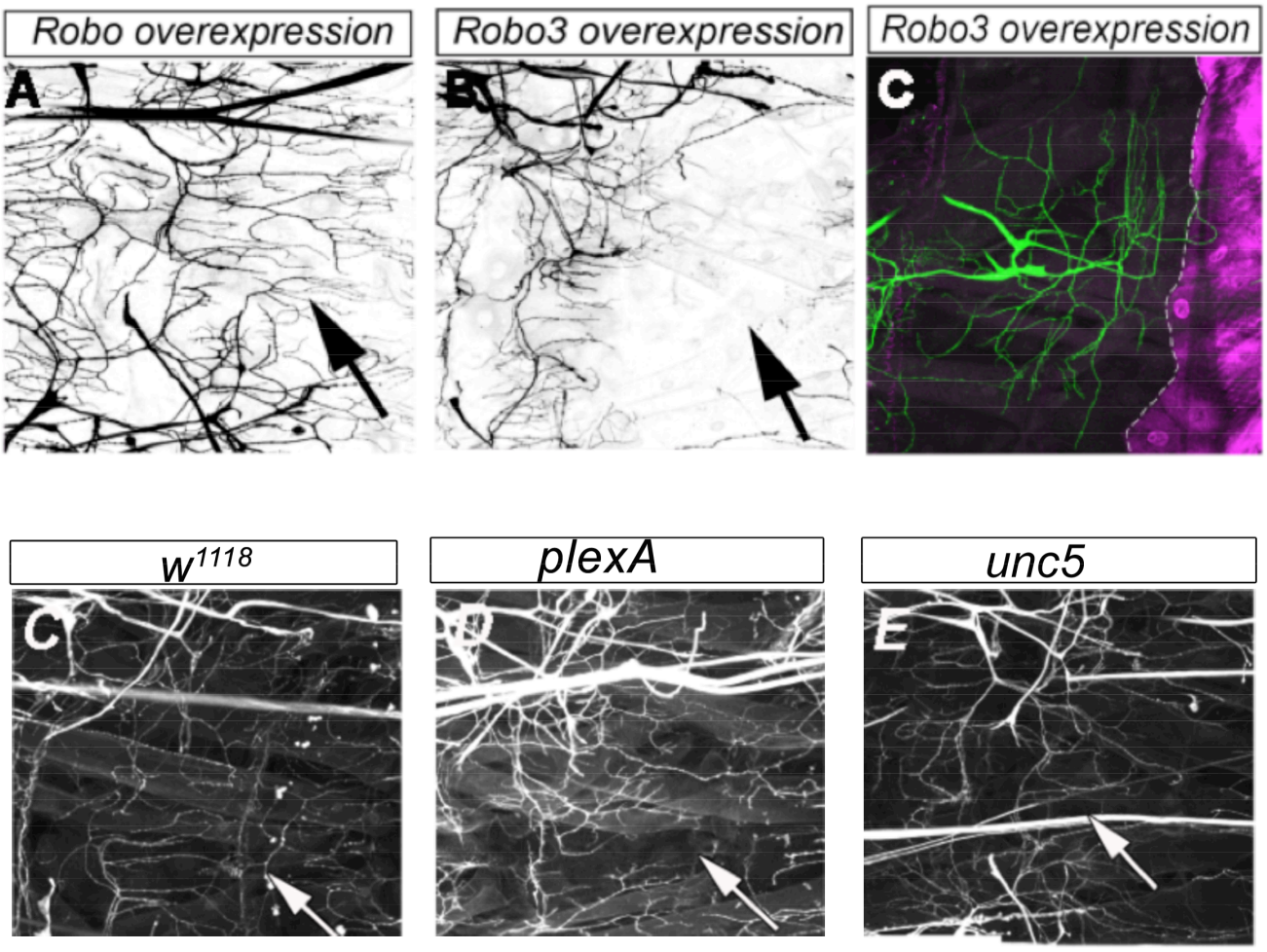


Figure 3.5 Robo3 receptor induces anterior compartment restriction

(A) Dendritic fields of da neurons labeled with *109(2)80 GAL4, UAS mCD8 :: GFP* expressing *UAS-robo*

(B) Dendritic fields of da neurons labeled with *109(2)80 GAL4, UAS mCD8 :: GFP* expressing *UAS-robo3*

(C) Dendritic fields of a class IV neuron labeled with *109(2)80 GAL4<CD2< UAS mCD8 :: GFP, en-lacZ* expressing *UAS-robo3.* Class IV dendrites labeled with GFP (green) and the p compartment labeled with LacZ (magenta)

(D) Dendritic fields of da neurons labeled with *109(2)80 GAL4, UAS mCD8 :: GFP*

(E) Dendritic fields of da neurons labeled with *109(2)80 GAL4, UAS mCD8 :: GFP* expressing *UAS-plexA*

(F) Dendritic fields of da neurons labeled with *109(2)80 GAL4, UAS mCD8 :: GFP* expressing *UAS-unc5*

Figure 3.6 Robo3 alone does not regulate class III tiling boundaries

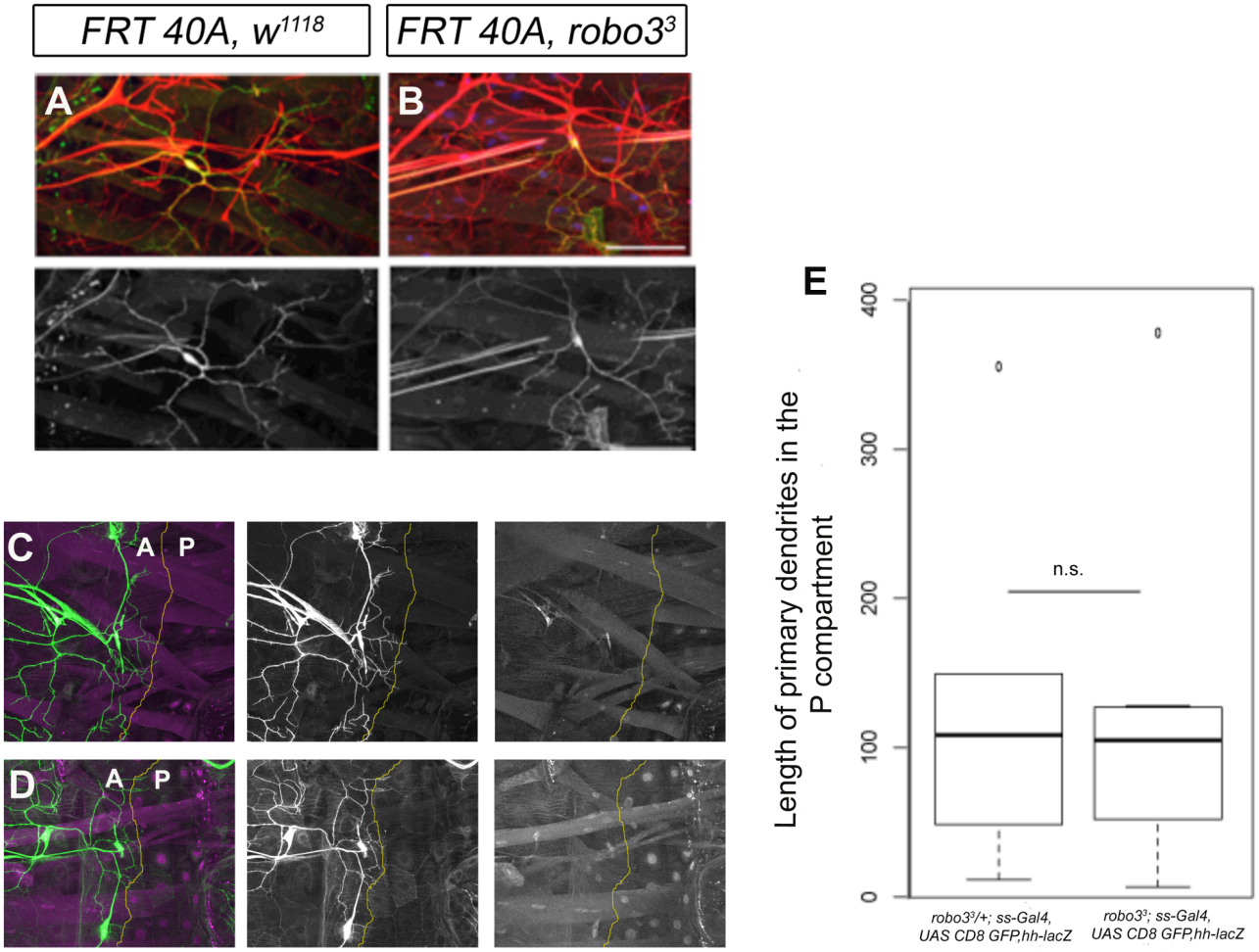


Figure 3.6 Robo3 alone does not regulate class III tiling boundaries

(A,B) Dendrites of vdaD in wildtype MARCM clones (left panel). Dendrites of vdaD *robo3* MARCM clones right panel

(C,D) Dendrites of vdaD in *robo3* mutant animals labeled with *ss-GAL4, UAS mCD8 ::GFP* and P compartment labeled with *hh-lacZ* . GFP(green), LacZ (magenta) Dendrites of vdaD labeled with *ss-GAL4, UAS mCD8 ::GFP* and P compartment labeled with *hh-lacZ* . GFP(green), LacZ (magenta)

(E) Quantification of the length of vdaD dendrites arborizing the P compartment

Figure 3.7 Commisureless is not sufficient for over-growth of class III neurons to the P compartment

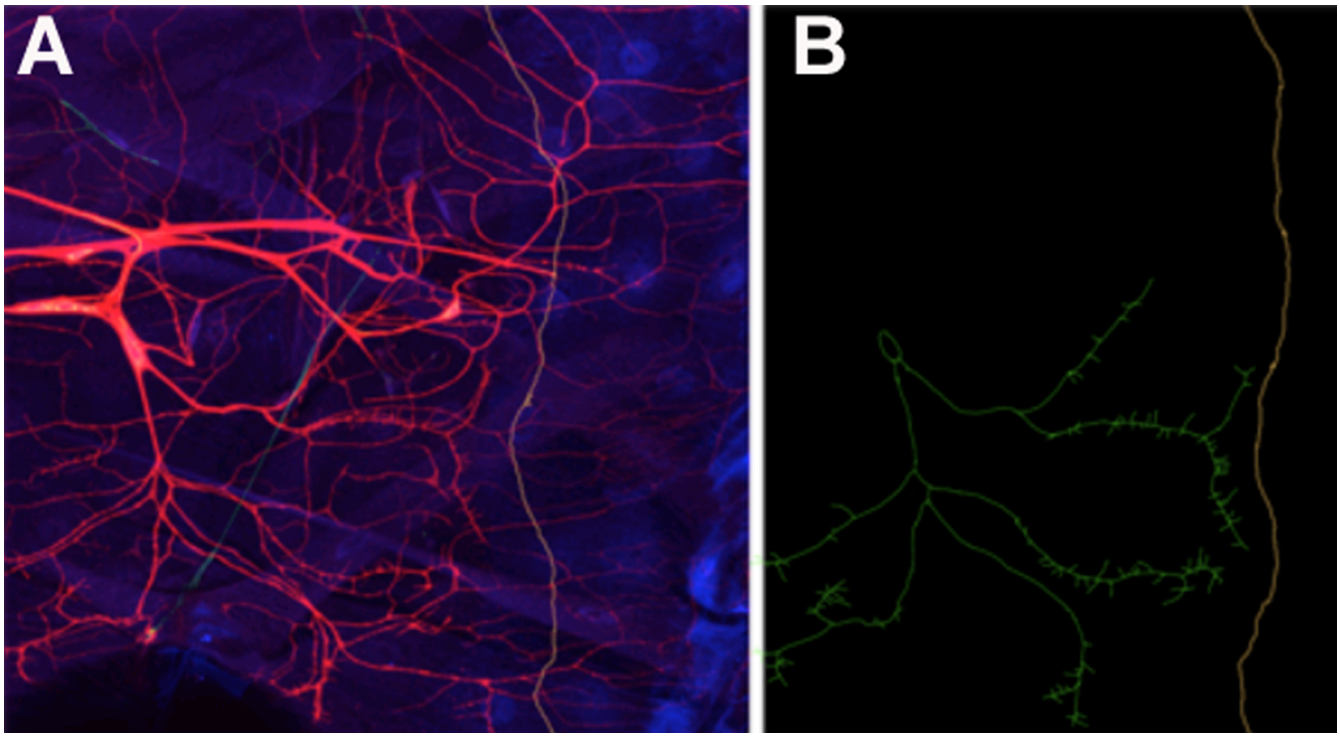


Figure 3.7 Commisureless is not sufficient for over-growth of class III neurons to the P compartment

(A) Dendritic fields of da neurons labeled with *109(2)80 GAL4, UAS mCD8 :: GFP* expressing *UAS-comm.* P compartment labeled with *en-lacZ* (blue)

(B) Tracing of the vdaD dendrites expressing *UAS-comm*

Figure 3.8 Knot prevents restriction of class III dendrites to the A compartment

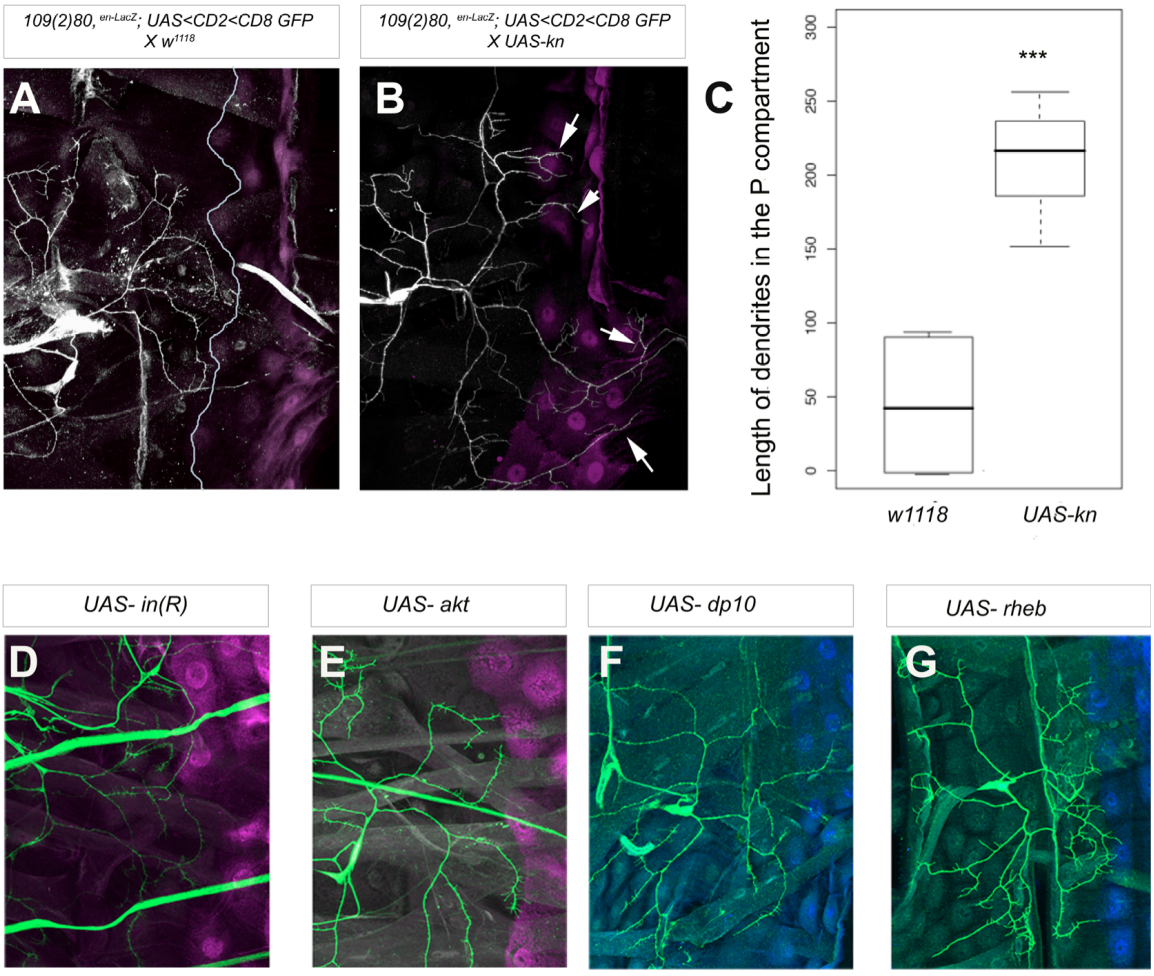


Figure 3.8 Knot prevents restriction of class III dendrites to the A compartment

(A) Dendritic fields of a vdaD labeled with *109(2)80 GAL4<CD2< UAS mCD8 :: GFP* and P compartment labeled with *en-lacZ* . vdaD dendrites labeled with GFP (white) and the P compartment labeled with LacZ (magenta)

(B) Dendritic fields of a vdaD labeled with *109(2)80 GAL4<CD2< UAS mCD8 :: GFP* expressing *UAS-kn* and P compartment labeled with *en-lacZ* . vdaD dendrites labeled with GFP (white) and the P compartment labeled with LacZ (magenta)

(C) Quantification of the total length of dendrites arborizing the P compartment

(D) Dendritic fields of a vdaD labeled with *109(2)80 GAL4<CD2< UAS mCD8 :: GFP* expressing *UAS-in(r)* and P compartment labeled with *en-lacZ* . vdaD dendrites labeled with GFP (green) and the P compartment labeled with LacZ (magenta)

(E) Dendritic fields of a vdaD labeled with *109(2)80 GAL4<CD2< UAS mCD8 :: GFP* expressing *UAS-akt* and P compartment labeled with *en-lacZ* . vdaD dendrites labeled with GFP (green) and the P compartment labeled with LacZ (magenta)

(F) Dendritic fields of a vdaD labeled with *109(2)80 GAL4<CD2< UAS mCD8 :: GFP* expressing *UAS-dp10* and P compartment labeled with *en-lacZ* . vdaD dendrites labeled with GFP (green) and the P compartment labeled with LacZ (blue)

(G) Dendritic fields of a vdaD labeled with *109(2)80 GAL4<CD2< UAS mCD8 :: GFP* expressing *UAS-rheb* and P compartment labeled with *en-lacZ* . vdaD dendrites labeled with GFP (green) and the P compartment labeled with LacZ (blue)

Figure 3.9 Class III dendrites do not avoid ectopic sources of Engrailed

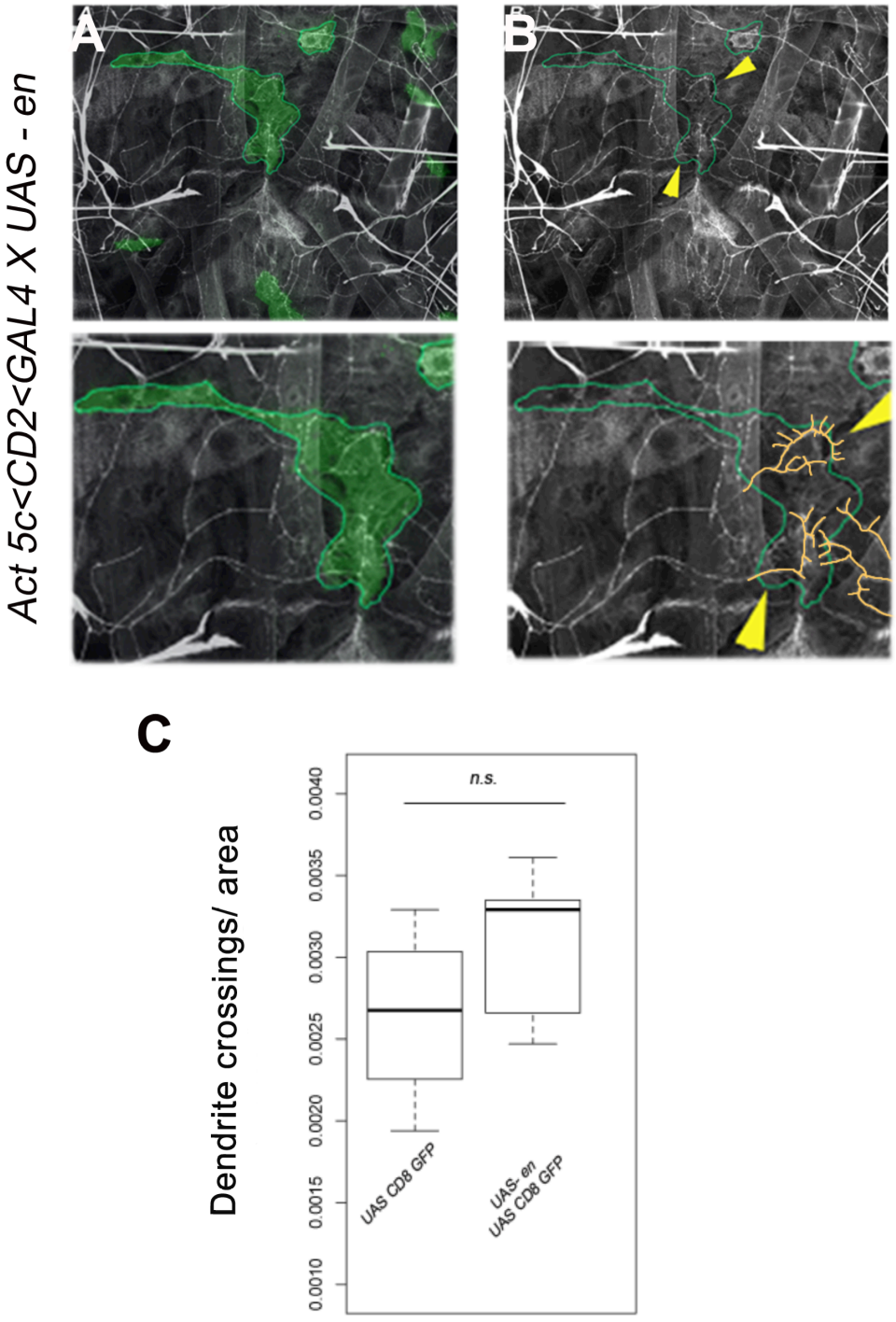


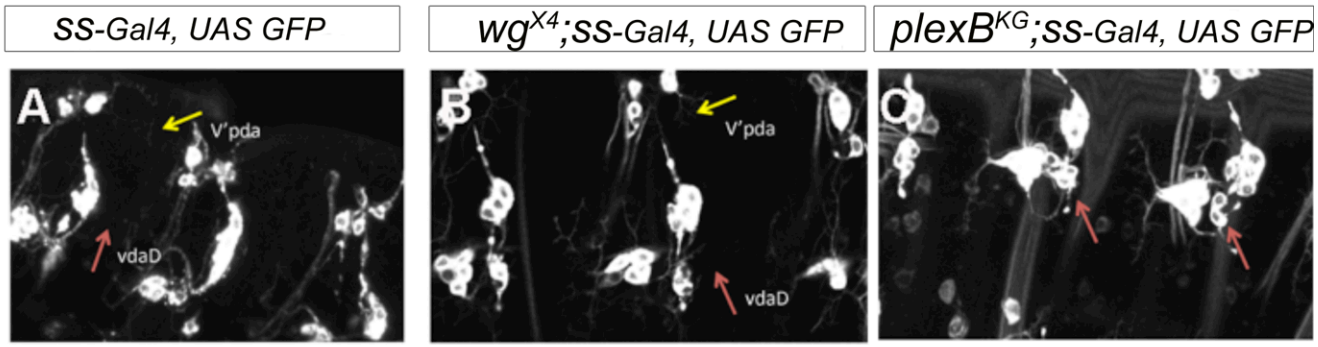
Figure 3.9 Class III dendrites do not avoid ectopic sources of Engrailed

(A) HRP immunoreactivity labels neuronal dendrites in *Act<5C<GAL4* larvae expressing *UAS- en* (green)

(B) Tracings of the boundary of *en* expressing cells. Dendrites labeled with HRP

(C) Quantification of the number of dendrites crossing/area

Figure 3.10 Classic boundary cues and signaling molecules do not affect the positioning of sensory neuron dendrites



D

Morphogen/ receptor/ ligand	Change in dendritic positioning
Patched (component of hh)	No (KO in class III)
Smothened (component of hh)	No (KO in class III)
Hedgehog	No (GOF in epidermis)
Wingless	No (GOF in epidermis/ whole mutant)
Engrailed	No (whole mutant)
NetAB	No (whole mutant)
Slit	No (whole mutant)
Ephrin	No (whole mutant)
Plexin B	No (whole mutant)
Sema 1a	No (whole mutant)

Figure 3.10 Classic boundary cues and signaling molecules do not affect the positioning of sensory neuron dendrites

(A) Dendritic fields of class III neurons labeled with *ss GAL4<CD2< UAS mCD8 :: GFP*

(B) Dendritic fields of class III neurons labeled with *ss GAL4<CD2< UAS mCD8 :: GFP* in wg^{X4} mutant embryos

(C) Dendritic fields of class III neurons labeled with *ss GAL4<CD2< UAS mCD8 :: GFP* in $plexB^{KG}$ mutant embryos

(D) Table of all the compartment cue and guidance molecules mutants tested for their role in class III dendrite restriction within the A compartment

Chapter 4

Potential functional role compartment restriction of sensory dendritic fields

Introduction

The territories that dendrites innervate determine the nature of the stimuli they receive. While a number of studies have sought to determine the molecular basis for territory boundary formation (Furrer et al, 2003; Furrer et al, 2007; Polleux et al, 2000), few studies have directly determined the functional consequences of specific dendritic boundaries. In this chapter, I address this link in the context of dendritic tiling.

Tiling refers to the complete and non-redundant coverage of input space by a population of neurons, and is a conserved mode of dendrite and axonal organization (Grueber et al, 2003b),(Grueber & Sagasti, 2010). Tiling has typically been studied in the context of functionally identical (homotypic) neurons, and it is assumed to be a way of ensuring that stimuli are received at any and all points within a receptive field. Prior studies in the Grueber lab, and work reported in Chapters 2 and 3, revealed a mode of tiling in the sensory system between functionally dissimilar neurons, which we termed heterotypic tiling. These findings raised new questions about the functional significance of this tiling organization, since it is likely established for different reasons than homotypic tiling. In this Chapter I asked how sensory dendritic field position might affect neuronal function. In order to understand the relationship between form and function, I performed live imaging studies and preliminary behavior tests.

Dendritic fields in the ventral cluster of da neurons show class specific relationships with epidermal compartment boundaries. Class I neurons grow in the P compartment, class II and III neurons grow exclusively in the A compartment, and class IV neurons show no obvious compartmental preference. Recent studies have shown that different classes of da neurons sample distinct stimuli (Grueber et al, 2007; Yan et al, 2013). Class IV neurons that innervate both the A and P compartment sense noxious stimuli. Class III neurons, which are restricted to the A compartment can sense gentle touch and Class I neurons primarily restricted to the P compartment provide proprioceptive feedback during larval locomotion (Hughes & Thomas, 2007) (Suster & Bate, 2002). Crawling requires integration of sensory input, motor neuron output and muscular activity. To elicit this simple behavior class I sensory neurons have to be correctly located to provide feedback to the motor neurons in the CNS, which then must synapse with the correct muscles to ensure normal crawling (Suster & Bate, 2002).

The restriction of dendritic fields of different classes of da neurons to specific compartments led us to ask whether this arrangement could relate to the function of these neurons. Why do dendritic fields of mechanosensory neurons avoid specific regions in the body wall? Prior studies together with our anatomical analysis of dendritic fields of da neurons led us to hypothesize that dendritic fields of different types of neurons should be restricted to specific parts of the body wall to ensure sampling of appropriate stimuli specific to their sensory modality, and possibly, so that they are not aberrantly activated.

Results

Transgenic markers for simultaneous live imaging of two classes of neurons

Anatomical studies from chapter 2 showed that dendrites of different classes are restricted to different regions of the epidermis. I wanted to examine how these specific innervation patterns might play a role in the effective sampling of stimuli by the sensory neuron dendrites. Previous studies have suggested that class I neurons and bipolar neurons provide proprioceptive feedback and class III neurons sense light touch (Hughes & Thomas, 2007), (Yan et al, 2013). In order to understand whether the restriction of dendritic fields is important for their sensory function, I first performed live imaging studies together with a rotation student Dan Costantini. To image class I and class III neurons simultaneously I first had to develop a system to label these two classes of neurons. I utilized the QF-QUAS system as it labels neurons independent of the GAL4-UAS system. recombined with *QUAS-td Tomato* (Yan et al, 2013; Zhang et al, 2013). This transgene gave me the ability to visualize the class III neurons in live animals. To simultaneously visualize the class I neurons I combined *NompC-QF, QUAS-td Tomato* with the class I Gal4 driver, *221 GAL4, UAS mCD8 :: GFP* (Fig. 4.1A).

Dendrites of all class I neurons undergo extensive distortion during larval crawling

We examined the behavior of the two classes of labeled neurons during larval crawling using live imaging on a multi-photon Nikon A1RMP microscope. Larvae were placed underneath a coverglass in a mixture of halocarbon oil, and occasionally a larva crawled across the objective in an orientation that allowed visualization of class I and III neurons in dorsal or ventral clusters. As the larva crawled the peristaltic wave propagated from the posterior to the anterior of the animal. We found that as the peristaltic wave approached a segment there was a slight segment lengthening, followed by contraction of muscles in the segment and folding of the cuticle. Notably, the folding contraction appeared to occur primarily within the field of the class I neuron (P compartment), while the field of the ventral class III neuron (A compartment) was largely unaffected by the peristaltic contraction. Closer examination revealed that the contraction of the P compartment during larval crawling always stopped when the contractions reached the primary dendrite of the ventral class I neuron, vpda (Fig. 4.1B,C). The longer secondary dendrites that extended within the P compartment were folded under the A compartment. Thus, during a peristaltic wave, folding of the ventral epidermis occurs primarily within the region of the proprioceptive class I neuron.

There are two other class I neurons, located in the dorsal cluster, that also provide proprioceptive feedback during crawling. To determine whether they also project dendrites to within regions of epidermal contortion we imaged animals that crawled across the objective in dorsal-up orientation. Perhaps because of the muscle pattern in the dorsal body wall, the effect of the peristaltic wave was distinct from that observed

ventrally. Here, I observed that the P compartment did not fold under the A compartment, but rather during segment contraction two folds, or "hinges" appeared in the cuticle. These hinges did not extend along the entire dorsal region of the animal, but instead were localized to a crescent of the dorsal body wall that overlapped precisely with location of the two class I neurons, ddaD and ddaE. Thus, despite their different positions and dendritic orientations, class I neurons commonly project dendrites to regions of the body wall that undergo the most consistent distortion during progression of the peristaltic wave.

Dendrites of ventral class III neurons undergo limited distortion during crawling

We next examined the behavior of the ventral class III neuron, vdaD, which tiles with vpda and innervates the A compartment. In contrast to vpda, vdaD likely functions, together with other class III neurons, as a light touch receptor. I observed that vdaD dendrites largely avoided contraction during the passage of a peristaltic wave except for the most distal tertiary dendrites at the AP compartment boundary. Although during crawling the A compartment undergoes a slight contraction, this did not lead to contraction or folding of vdaD dendrites as the part of the A compartment that contracts is not innervated by vdaD dendrites. Thus, in contrast to class I dendrites, the dendrites of the ventral class III neuron does not undergo extensive distortion or movement during passage of the peristaltic wave.

Silencing class I neurons disrupts crawling behavior of neurons

From the results of our previous live imaging, we hypothesized that contraction led to the activation of class I neurons that provided the nervous system with positional information during crawling. We reasoned that if we silenced the class I neurons, crawling speed would be affected as they would not provide appropriate proprioceptive feedback. In order to silence the class I neurons, I used a class I driver *221 GAL4* and specifically expressed tetanus toxin in them using *UAS-tnt* (Sweeney, 1995; Suster & Bate, 2002). Tetanus toxin cleaves the synaptic vesicle protein Synaptobrevin and as a result blocks neurotransmitter release. As a control I ectopically expressed the inactive form of tetanus toxin under the control of *221 GAL4*. In order to assess if there were any changes in larval crawling behavior between animals in which the class I neurons were silenced and the controls, I used speed (distance/time) as a parameter to assess potential crawling defects. As I hypothesized, animals in which the class I neurons were silenced crawled significantly slower than the larvae in which class I neurons were active (Fig. 4.2A).

One caveat of using *221 GAL4* is that it also expressed in cells in the brain lobes and ventral nerve cord (VNC). To ensure that the change in speed was not a result of silencing motor neurons, I repeated the experiments and added *teashirt* (tsh)-*GAL80*, which silences *GAL4* activity in the VNC where motor neuron cell bodies are located. It is worth noting that previous larval crawling studies (Suster & Bate, 2002) have shown that neurons in the brain lobe do not affect crawling speed and behavior of larvae. Similar to the results without *tsh-GAL80*, specific silencing of class I neurons in the PNS resulted in significantly slower crawling speeds in larvae compared to larvae in which

class I neurons were not silenced (Fig. 4.2b). These results suggest that silencing of class I neurons leads to a significant decrease in crawling speeds in larvae.

Change in the position of primary dendrite of vpda does not affect larval crawling speed

Our results showed that class I neurons were important for normal larval crawling speed and that during crawling the contraction of the segment terminated at the point at which the primary dendrite was visible and all the secondary dendrites folded with or underneath the contracted epidermis and muscles. These results together led me to ask whether the position of the primary dendrite influenced larval crawling and if shifting the primary dendrite would change the crawling speed of larvae. In order to address this question, I shifted the position of the primary dendrite of vpda by misexpressing *hh* in the Wg compartment, which I knew from experiments presented in chapter 3 would induce a posterior shift of the primary dendrite position of vpda. I hypothesized that if the position of the vpda primary dendrite was important for providing appropriate proprioceptive feedback, then shifting the position of the primary dendrite may decrease the crawling speed of the larvae. I assessed the crawling speed of larvae where the primary dendrite was shifted into the P compartment and compared to the crawling of speed of larvae where the primary dendrite was present along the AP compartment boundary. I found no significant change between their crawling speeds (*wt* n=20, *UAS-hh* = 18, p= 0.84). These preliminary results suggest that the modest positional shift of the vpda primary dendrite caused by Hh misexpression does not affect the normal crawling speed of larvae (Fig. 4.3).

Discussion

The da neurons are sandwiched between the peripheral muscles and the epidermis (Jan & Jan, 2010). In addition, *Drosophila* larvae muscles are arranged in a precise pattern within a segment and the muscle attachment sites of ventral muscles correlate with the AP compartment boundary. One outstanding question that still remains is whether the nature of muscle contraction varies between compartments, and if so, does it correlate with the function of neurons specific to those compartments? Our live imaging studies suggest that the A and P compartment do not contract to the same extent during locomotion. As a peristaltic wave passes through a segment during crawling, the P compartment shows a nearly complete folding contraction whereas the A compartment shows only a slight fold in the middle of the compartment. Thus, the dendritic fields of vdaD are restricted to the A compartment where it does not undergo contraction during crawling, while the dendritic fields of vpda are restricted to the P compartment and fold under the P cells during larval movement. These observations correlate well with the different activation thresholds of these neurons. Class III neurons sample gentle stimuli such as touch and may be aberrantly activated if they are exposed to mechanical disturbances during the passage of a peristaltic wave while crawling. In the case of vpda neurons that provide positional information, these neurons may perceive the most contraction, which leads to their activation and provides appropriate proprioceptive feedback about the status of the larval segment during crawling (Fig. 4.4). Furthermore, silencing of class I neurons affects crawling behavior suggesting that they provide

sensory input necessary for larval crawling. This result differs from the findings from previous studies examining the behavior of class I neurons, where class I neurons and bipolar neurons functioned redundantly and silencing either one was not sufficient to alter larval crawling behavior (Hughes et al, 2007).

Functional consequences of spatial restriction of dendritic fields

Dendrites receive input from their environment. In the case of sensory dendrites, the input may take the form of sensory deformation of the body wall, olfactory cues from the air, or noxious thermal cues. Most sensory cues are unpredictable in their origin, and thus allow animals to respond effectively to a changing external environment. For example, noxious stimuli could in principle land anywhere along the body wall at any time and thus it makes functional sense for nociceptor dendrites, such as class IV neurons, to cover the entire body wall completely. By contrast, some sensory inputs may be highly predictable. One perfect example would be the motion of the body wall during repetitive peristaltic movements. In these cases, sensory function could be optimized if sensory dendrites are targeted precisely to regions of the body wall that can most effectively and reliably sense these movements. Another case could be envisioned, in which reception of predictable sensory input is not desirable, so that detection by certain neurons is to be avoided so that they may be free to detect other stimuli.

During larval crawling a peristaltic wave propagates from the posterior part of the animal to the anterior part and propagation of the wave causes the segment to contract. Our live

imaging studies have demonstrated that there is a difference in the extent of contractions that the A and P compartment undergo. This is likely attributed to the different muscle attachment patterns in the A and P compartment. The difference in the extent of contraction between A and P compartments was especially interesting due to the compartment-specific arrangement of the da neurons. Class III neurons sense gentle touch (low threshold) and reside in the A compartment (Yan et al, 2013). Class I neurons are proprioceptive and are restricted to the P compartment. The results from live imaging studies have led me to propose that class III neurons are restricted to the A compartment so that they do not experience the same level of mechanical movement compared to the P compartment, and thus do not become aberrantly activated during crawling. By contrast, class I neuron dendrite position is likely positioned to receive reliable input from portions of the body wall that contract with each peristaltic wave. Notably, neurons that are not restricted to one compartment or another are the high threshold nociceptors. It follows that these neurons are not going to be aberrantly activated by simple stretch.

These assertions are based on anatomical and live imaging studies but fit well with functional studies of sensory neurons conducted to date (Stewart et al, 2012; Yan et al, 2013),(Hughes & Thomas, 2007). There are many experimental predictions that follow from this proposed scenario. For one, calcium imaging of wildtype class III neurons and neurons in which their dendrites extend into the P compartment upon misexpression of kn could be informative. This experiment will be informative about whether the restriction of class III dendrites to the A compartment prevents aberrant activation of class III neurons. If our hypothesis is correct then wildtype class III neurons should remain

inactive during crawling and those misexpressing *kn* should be activated by the peristaltic wave.

Role of precise dendrite positioning in larval behavior

During crawling, the contraction in the P compartment halts when it reaches the primary dendrite of class I neurons. These contractions likely activate the class I neuron, which provides proprioceptive feedback to ensure normal crawling behavior. Indeed, I confirmed previous findings that silencing of class I neurons disrupts crawling behavior as the larvae crawled significantly slower when compared to wild-type (Hughes & Thomas, 2007). My previous studies indicate that the position of the primary dendrite is highly regulated, but why is there such a strong correlation between the position of the primary dendrites and the location at which the peristaltic wave terminates? I can envision a few scenarios that when the peristaltic wave reaches the primary dendrite it activates the neuron, which then provides proprioceptive feedback and allows coordinated crawling behavior. Alternatively, the primary dendrites, on sensing the wave, provide sensory input that translates into the termination of muscle contractions responsible for propagating the wave.

I tested whether the position of the primary dendrite position was important for normal crawling behavior. Contrary to my hypothesis the change in position did not alter crawling speed. However, this lack of change does not imply that primary dendrite position is not important for proprioception. Previous studies have shown that crawling

is controlled by central pattern generators (CPG) can occur in the absence of sensory input. However, to ensure coordinated crawling behavior, appropriate sensory input needs to be integrated into CPG circuitry (Suster & Bate, 2002). Thus, in the absence of appropriate sensory input, the larvae may still crawl at normal speeds but there might be a disruption in the normal coordinate muscle contractions. Similarly, changing the primary dendrite position of vpda may not disrupt crawling speed but may disrupt other aspects of crawling such as coordinated muscles contractions.

Summary

Homotypic tiling ensures complete and non-redundant coverage of the receptive areas by cells of similar functions. I propose that heterotypic tiling enables proper arrangement of dendritic fields in a cell-type specific manner ensuring that they receive and respond to stimuli that are unequally distributed across the body wall. It is possible that during locomotion (larval crawling), parts of the body wall contort differently, which leads to the dendritic fields of some neurons to fold into the body wall while other are still on the surface to receive stimuli. Thus, to ensure that dendrites of neurons belonging to specific modalities can receive stimuli, which may be important during locomotion, arbors of different types of neurons are distributed by robust molecular mechanisms to appropriate parts of the body wall.

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Figure 4.1 Dendrites of all class I neurons undergo extensive distortion during larval crawling

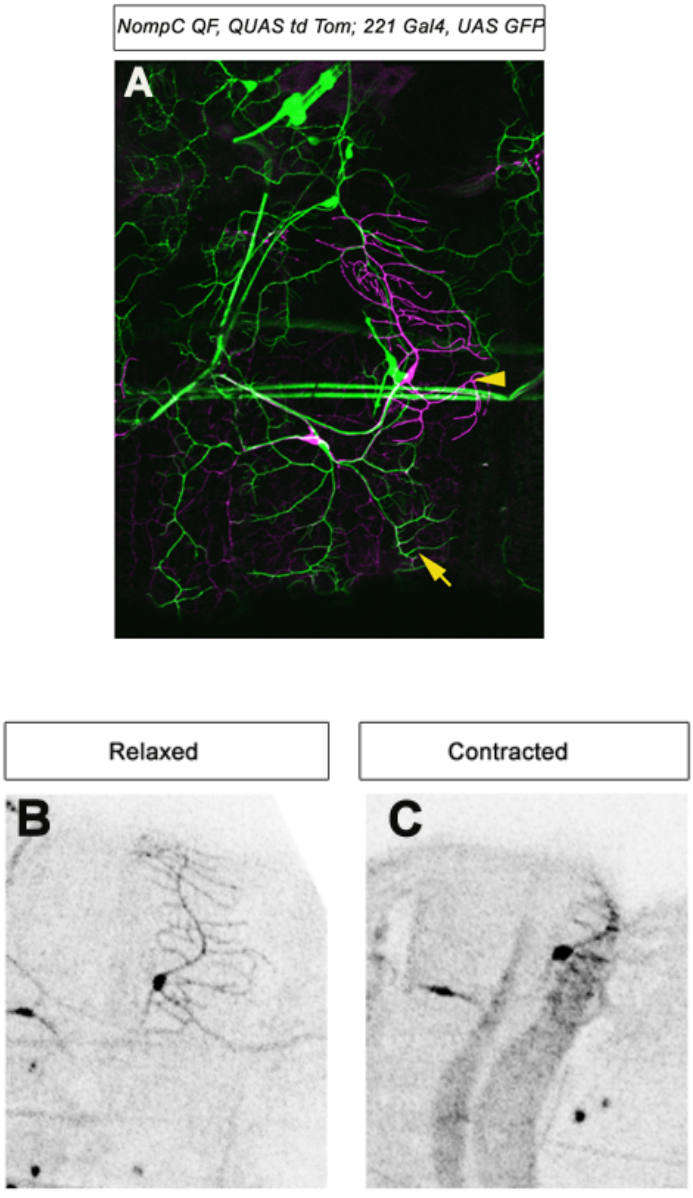

Figure 4.1 Dendrites of all class I neurons undergo extensive distortion during larval crawling

(A) *NompC QF, QUAS tdTom* was used to visualize class III dendrites and *221 Gal4, UAS CD8 GFP* was used to visualize class I dendrites live.

(B) Represents vpda in a relaxed state during the propagation of a peristaltic wave

(C) Represents vpda in a contacted state during the propagation of a peristaltic wave

Figure 4.2 Silencing class I neurons affects larval locomotion

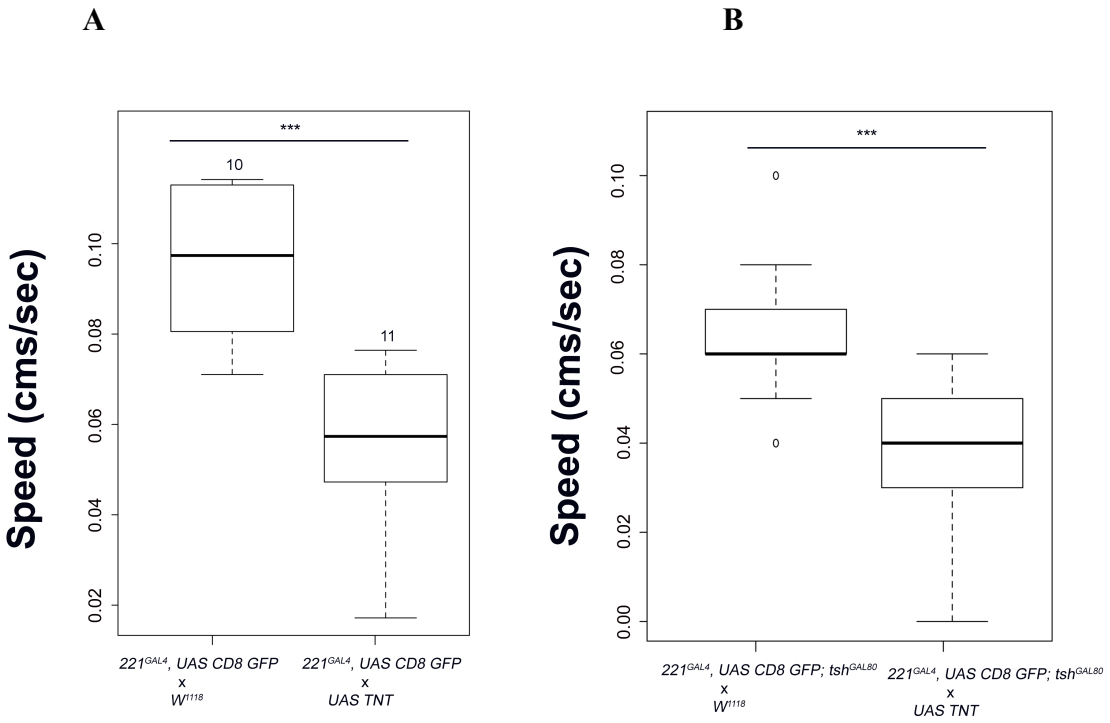

Figure 4.2 Silencing class I neurons affects larval locomotion

(A) *221 Gal4, UAS CD8 GFP* flies were crossed *UAS-tnt* and w^{1118} to assess the speed of class I in larval crawling. The box plots represent the average crawling speed

(B) *221 Gal4, UAS CD8 GFP; tsh-Gal80* flies were crossed *UAS-tnt* and w^{1118} to assess the speed of class I in larval crawling. The box plots represent the average crawling speed.

Figure 4.3 Shift in primary dendrite position does not affect crawling speed

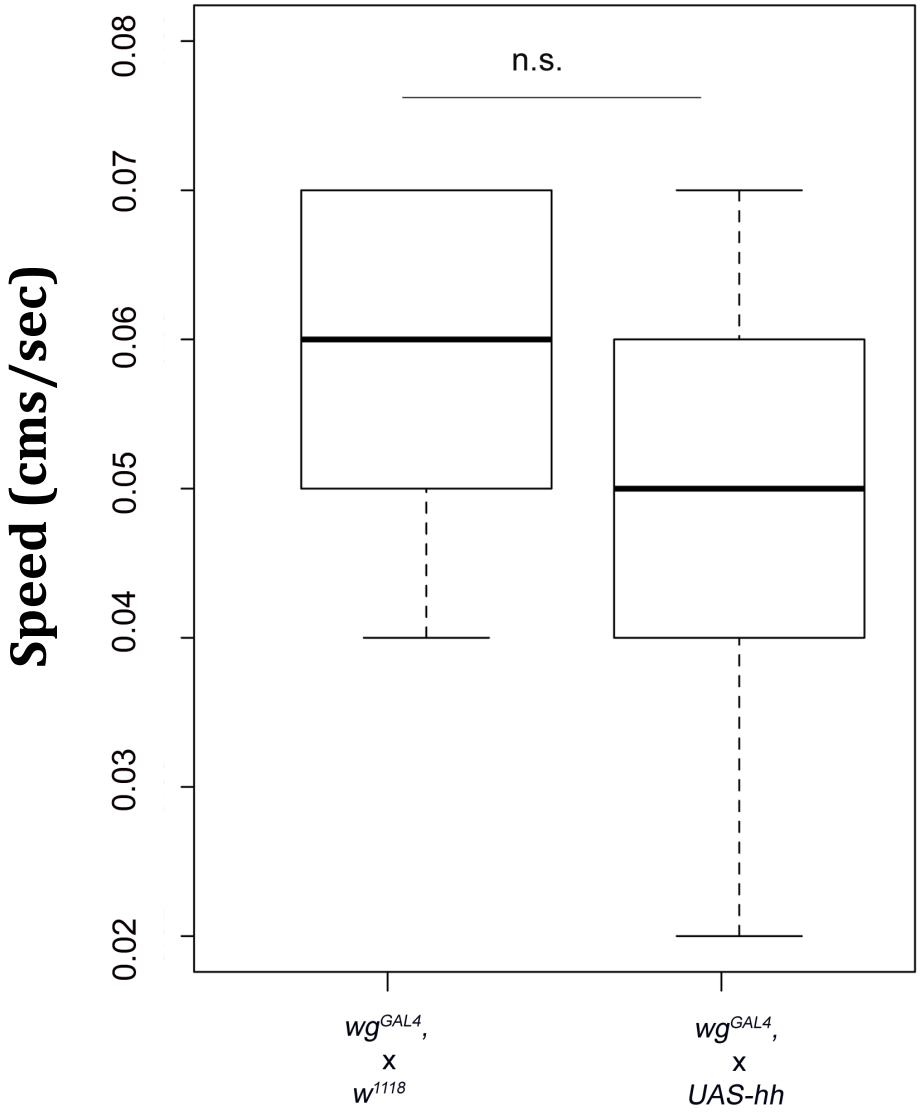

Figure 4.3 Shift in primary dendrite position does not affect crawling speed

Wg-Gal4 flies were crossed to UAS-hh and w^{1118} flies to the shift the primary dendrite position of vpda neurons. The crawling speeds were assessed of both sets of larvae. The box plots represent the average crawling speeds of larvae.

Conclusions and Future Directions

Dendrites develop in highly complex environments and their interactions with neighboring neurons and the substrate are thought to be important for the establishment of their dendritic territories. Mechanisms required for the establishment of dendritic territories remain largely elusive. This thesis investigates the role of both dendrite-dendrite interactions and dendrite-substrate interactions in determining dendritic boundaries. As a part of this study I have uncovered novel mechanisms that neurons utilize to define dendritic borders and how those borders may be important determinants of neuronal function. The experiments described in this thesis have taken advantage of genetic tools in *Drosophila* that allow manipulation and visualization of individual neurons.

Novel mechanisms establish homotypic tiling

It is now well established that class IV neurons establish their dendritic territories by engaging repulsive dendrite-dendrite interactions with neighboring class IV neurons (Grueber et al, 2003b). For these repulsive interactions to occur, it is necessary that dendrites of neighboring neurons come into physical contact (Lawrence Zipursky & Grueber, 2013). However, dendritic fields of the other populations of da neurons are not contiguous and there are often significant gaps between the boundaries of their fields. Thus, additional mechanisms must exist that determine dendritic fields of other classes of neurons (Grueber et al, 2003b). My ablation experiments carried out on class III neurons indeed suggest that contact-dependent repulsive interactions are only required for refining the dendritic fields of class III neurons. Thus, novel tiling mechanisms must exist that determine the tiling borders of dendritic fields. Tiling boundaries may be determined by

a combination of extrinsic cues from the body wall and interactions with dendrites of different da neuron classes. Evidence for additional tiling cues have been supported by studies in vertebrates, where genetic ablation of RGC in mice does not result in invasion of the vacated space by neighboring neurons (Lin et al, 2004).

Anatomical studies revealed that tiling boundaries can exist between dendritic fields of neurons belonging to different functional classes. The existence of heterotypic tiling borders challenges the idea that tiling is solely, or primarily, a mechanism that prevents non-redundant coverage of functionally similar neuronal fields. The possible functional significance of this observation is discussed in greater detail below. In addition my observations suggest that neurons belonging to different classes have mechanisms by which they can recognize each other and that this recognition may restrict dendritic boundaries. These findings suggest that heterotypic tiling is an alternative way in which dendritic borders are defined, but left the major mechanism for heterotypic tiling undefined. Although ablation of neurons that engage in heterotypic tiling did result in excessive branching in distal dendrites, the manipulation did not alter the dendritic boundary of the remaining neuron, suggesting dendritic boundaries are determined by additional cues.

These additional cues may be present in the substrate, given that prior work from the lab showed that dendritic territories correlate with molecular boundaries present in the epidermis that divide the *Drosophila* embryonic segments into A and P compartments. Different classes of da neurons show specific compartment preferences, class II/III dendrites are restricted to the A compartment, class I neuron dendrites are mostly

restricted to the P compartment, and class IV dendrites show no clear compartmental preferences. Similar to sensory neuron dendrites, the territories of motor neuron dendritic arbors in the CNS also obey A and P compartment boundaries (Landgraf et al, 2003). The molecular basis of motor neuron dendrite positioning is not known, but this coincidence raises the possibility that similar patterning cues may operate in the CNS and PNS, possibly functioning to bring central and peripheral arbors into spatial register with one another. This raises the interesting question of whether da neuron axons obey the same class-specific compartment rules as dendrites. The morphologies of all da neuron axons have been characterized, and further analysis to ask whether axon terminals show A or P compartment restriction will be important for understanding whether compartmental cues might operate at several levels to organize sensorimotor circuitry.

Morphogens define molecular boundaries in the body wall and are essential to the development of the epidermis. However, removal of these signaling molecules do not show any obvious disruption of class III dendritic territories. Thus, for class III dendrite territory formation, known compartmental cues could either play no role in boundary formation, or could be redundant with repulsive cues acting between class I and class III neurons. Removal of both molecular cues and the tiling neuron would be required in order to test this hypothesis. These experiments should be a subject of future studies and will be important for uncovering cues that regulate dendritic restriction. Another way to uncover the underlying mechanisms and identify potential novel receptors required to identify the extrinsic signal-restricting class III dendritic boundary would be to take an unbiased approach and examine the consequences of loss of candidate cell surface

molecules for class III arborzation in larvae in which class I neurons are missing, such as in *ato* mutants.

Potential novel mechanism of Hedgehog signaling in dendrites

In addition to investigating dendro-dendritic and dendrite-substrate interactions that specify class III dendritic boundaries, I also explored the extrinsic signals that govern class I dendritic territories. As the class I dendritic territory correlated positively with Hh expression, I performed gain and loss of function studies with Hh, two receptors that transduce the Hh signal, Ptc and Smo, and the TF downstream of Hh, Ci (Lawrence & Struhl, 1996),(Chen & Struhl, 1998). My experiments revealed that removal of *ptc*, *smo* and over-expression of a dominant negative form of Ci specifically in the class I neuron vpda caused a shift in the positioning of the primary dendrite. Normally the primary dendrite of vpda is located along the AP compartment boundary, whereas in these mutants it grows into the P compartment. These results lead to two important, and unexpected, conclusions. First, disrupting Hh signal components causes a shift in the primary dendrite, but the overall territory of class I dendrites remained unchanged, suggesting that different parts of a single dendritic arbor are regulated separately by Hh signaling. This raises exciting new possibilities of how and why distinct branches of dendrites respond differently to the same signal. Potentially, primary and secondary dendrites are responding to distinct sensory stimuli and separate regulation of their fields is essential for producing morphologies that are "tuned" to different directional stimuli. Second, unlike previous experiments, Ptc and Smo acted in a complimentary manner to regulate primary dendrite position along the AP compartment boundary. These results

have led me to propose a model in which the levels of Ptc on the cell surface are important for determining primary dendrite position. Previous studies have shown that Ptc is required for both the transduction and sequestration of Hh (Chen & Struhl, 1996; Lawrence et al, 1999). Initially, Hh binds to Ptc to transduce the Hh signal, which leads to the transcription of *ptc* along with many other target genes. This results in higher levels of Ptc on the cell surface such that the amount of Ptc on the cell exceeds the amount of Hh outside leading to the de-activation of the Hh pathway. Transcription of *ptc* upon Hh transduction requires Smo and *smo* mutants would be unable to transcribe additional *ptc* upon exposure to the Hh. Thus, in both *ptc* and *smo* mutants no additional *ptc* is transcribed, which results in a low Ptc cell. If levels of Ptc are important for regulating primary dendrite position, then removal of both *ptc* and *smo* should lead to the mis-positioning of the primary dendrite. My results fit well with this model and suggest that the levels of Ptc are critical for positioning the primary dendrite along the AP compartment boundary. Furthermore, expression of *ptc-loop2* (a *ptc* mutant that lacks the ability to bind to Hh) pan-neuronally (*Lee, J. and Grueber, WB. unpublished results*) does not disrupt primary dendrite position, which leads me to propose that Ptc may regulate primary dendrite positioning in a Hh-independent manner. In order to test this idea, it would be important to test whether *ptc* mutant neurons can be rescued with *ptc-loop2* transgenes. If the primary dendrite position is restored in this experiment, it would suggest that Ptc can function in a Hh-independent manner. This finding would reveal a novel role for a receptor that is a part of a highly conserved signaling pathway. If Ptc can function in a Hh-independent manner, does it then bind to another ligand or can it act in a

ligand-independent manner? Also, what is the downstream signaling machinery that is required for this function? These questions require further investigation and will be important for understanding how the same receptor can be re-utilized for diverse developmental processes.

Functional implications of A-P compartment restriction

My studies, and previous results from the lab, also raise questions about the functional significance of restricting dendritic fields of mechanosensory neurons to specific regions within the body wall. At a fundamental level, the locations of dendritic fields determine the regions from which neurons will sample sensory input. Our live imaging studies showed that during larval crawling, A and P compartments contract differently. Epidermis in the P compartment undergos almost complete contraction while epidermis in the A compartments only deforms slightly with each peristaltic wave. Interestingly, low threshold touch receptors neurons lie mostly in the A compartment and do not undergo the massive contractions experienced by proprioceptive neurons that are located in the P compartment. It may be that compartmental restrictions ensure a) that low threshold neurons are not aberrantly activated due to the contractions resulting from the peristaltic wave of the crawling larvae, and b) that proprioceptive neurons are positioned to best detect the status of the peristaltic wave. This hypothesis is supported by prior studies showing that proprioceptive neurons provide feedback to the CNS (Hughes & Thomas, 2007) about peristaltic movements, and my studies showing that silencing of proprioceptive neurons resulted in a disruption of crawling behavior and slower crawling speeds.

Whether the positioning of proprioceptive dendrites influences crawling speed is still uncertain. When the P compartment contracts during peristalsis, the distortion of the epidermis does not reach beyond the position of the primary dendrite at the AP compartment boundary, suggesting its position may be important for providing feedback. However, preliminary studies indicated that changing the position of this dendrite by overexpressing Hh in the Wg domain did not alter larval crawling speed. These results do not rule out the possibility that primary dendrite position may affect different aspects of crawling behavior. Further live imaging studies where the primary dendrite of proprioceptive vpda neuron is shifted will be important in deciphering whether changes in primary dendrite position alters the manner in which the peristaltic wave is sensed and propagated. Conducting these experiments will be challenging as both the P compartment and the class I neurons need to be labeled while perturbing the primary dendrite position. Furthermore, calcium imaging studies on crawling larvae could help to determine whether contractions in the P compartment are necessary for activation of class I neurons, and if extensive invasion of class III dendrites into the P compartment result in their aberrant activation during crawling. In addition, sensitive behavior assays need to be developed to test whether changing primary dendrite position affects burrowing, foraging and escape behaviors.

Together, these findings provide new insights into the mechanisms of dendritic territory formation and advance our understanding of how complex neural circuits are assembled during development in highly regulated ways likely to promote robust behavioral outputs.

Hopefully these studies will encourage further work in this system that might reveal additional insights into these key problems.

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